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| <p>(54) Title: IMMOBILIZED SEQUENCE-SPECIFIC PROBES</p> <p>(57) Abstract</p> <p>An improved nucleic acid hybridization assay reagent capable of binding a nucleic acid having a selected target sequence comprises a solid support matrix having oligonucleotide probes covalently attached thereto via a spacer arm. In a preferred embodiment, the solid support has reactive amino groups capable of binding UV irradiated nucleotides in an oligonucleotide probe, which probe comprises a hybridizing region complementary to a target sequence to be detected and a linking tail, the tail being composed of nucleotides, one or more of which is covalently bonded to the reactive groups of said support.</p> | | |

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IMMOBILIZED SEQUENCE-SPECIFIC PROBES

This invention relates to nucleic acid chemistry and to methods for detecting particular nucleic acid sequences. More specifically, the invention relates to a method for immobilizing DNA and RNA probes, stable assay reagents comprising the immobilized probes, and hybridization assays conducted with these immobilized probes. The invention has applications in the fields of medical diagnostics, medical microbiology, forensic science, environmental monitoring of microorganisms, food and drug quality assurance, and molecular biology.

Investigational microbiological techniques have been applied to diagnostic assays. For example, Wilson *et al.*, U.S. Patent No. 4,395,486 discloses a method for detecting sickle cell anemia by restriction fragment length polymorphism (RFLP). Wilson *et al.* identified a restriction enzyme capable of cleaving a normal globin gene but incapable of cleaving the mutated (sickle cell) gene. As sickle cell anemia arises from a point mutation, the method is effective but requires 10 to 20 ml of blood or amniotic fluid.

Various infectious diseases can be diagnosed by the presence in clinical samples of specific DNA sequences characteristic of the causative microorganism or infectious agent. Pathogenic agents include certain bacteria, such as Salmonella, Chlamydia, and Neisseria; viruses, such as the hepatitis, HTLV, and HIV viruses; and protozoans, such as Plasmodium, responsible for malaria. U.S. Patent No. 4,358,535 issued to Falkow *et al.* describes the use of specific DNA hybridization probes for the diagnosis of infectious diseases. The Falkow *et al.* method for detecting pathogens involves spotting a sample (e.g., blood, cells, saliva, etc.) on a filter (e.g., nitrocellulose), lysing the cells, and fixing the DNA through chemical denaturation and heating. Then, labeled DNA probes are added and allowed to hybridize with the fixed sample DNA, and hybridization indicates the presence of the pathogen DNA. A problem inherent in the Falkow *et al.* procedure is insensitivity; the procedure does not work well when very few pathogenic organisms are present in a clinical sample from an infected patient or when the DNA to be detected constitutes only a very small fraction of the total DNA in the sample. Falkow *et al.* do teach that the sample DNA may be amplified by culturing the cells or organisms in place on the filter.

Routine clinical use of DNA probes for the diagnosis of infectious diseases would be simplified considerably if non-radioactively labeled probes could be employed as

described in EP 63,879 to Ward. In the Ward procedure, horseradish peroxidase (HRP) labeled DNA probes are detected by a chromogenic reaction similar to ELISA. The Ward detection methods and reagents are convenient but relatively insensitive, again because the specific sequence that must be detected is usually present in extremely small quantities.

5 A significant improvement in DNA amplification, the polymerase chain reaction (PCR) technique, was disclosed by Mullis in U.S. Patent No. 4,683,202, and detection methods utilizing PCR are disclosed by Mullis *et al.* in U.S. Patent No. 4,683,195. In the PCR technique, short oligonucleotide primers are prepared which match opposite ends of a sequence to be amplified. The sequence between the primers need not be known. A sample of DNA or RNA is extracted and denatured, preferably by heat. Then,
10 oligonucleotide primers are added in molar excess, along with dNTPs and a polymerase, preferably Taq polymerase, which is stable to heat and commercially available from Perkin-Elmer/Cetus Instruments. DNA polymerase is "primer-directed," in that replication initiates at the two primer annealing sites. The DNA is replicated, and then again denatured.

This replication results in two "long products," which begin with the respective
15 primers, and the two original strands (per duplex DNA molecule). The products are called "long products," only because there is no defined point of termination of the synthesized strand. The reaction mixture is then returned to polymerizing conditions (e.g., by lowering the temperature, inactivating a denaturing agent, and, if necessary, adding more polymerase), and a second cycle initiated. The second cycle provides the two original
20 strands, the two long products from cycle one, two new long products (replicated from the original strands), and two "short products" replicated from the long products produced in cycle one. The products are called "short products," because these strands must terminate at the 5' end of the "long product" template — the end defined by the primer that initiated synthesis of the long product. The short products contain the sequence of the target
25 sequence (sense or antisense) with a primer at one end and a sequence complementary to a primer at the other end. On each additional cycle, an additional two long products are produced, and a number of short products, equal to the number of long and short products remaining at the end of the previous cycle, are also produced. Thus, the number of short products can double with each cycle. This exponential amplification of a specific target
30 sequence allows the detection of extremely small quantities of DNA.

The PCR process has revolutionized and revitalized the nucleic acid based medical diagnostics industry. Because the present invention provides reagents that will often be

utilized in conjunction with PCR, some additional background information on PCR may be helpful. The PCR process can be used to amplify any nucleic acid, including single or double-stranded DNA or RNA (such as messenger RNA), nucleic acids produced from a previous amplification reaction, DNA-RNA hybrids, or a mixture of any of these nucleic acids. If the original or target nucleic acid containing the sequence variation to be amplified is single stranded, its complement is synthesized by adding one or more primers, nucleotides, and a polymerase; for RNA, this polymerase is reverse transcriptase.

The PCR process is useful not only for producing large amounts of one specific nucleic acid sequence, but also for amplifying simultaneously more than one different specific nucleic acid sequence located on the same or different nucleic acid molecules. When one desires to produce more than one specific nucleic acid sequence in PCR, the appropriate number of different oligonucleotide primers are utilized. For example, if two different specific nucleic acid sequences are to be produced, four primers can be utilized: two for each specific nucleic acid sequence to be amplified.

The specific nucleic acid sequence amplified by PCR can be only a fraction of a larger molecule or can be present initially as a discrete molecule, so that the specific sequence amplified constitutes the entire nucleic acid. In addition, the sequence amplified by PCR can be present initially in an impure form or can be a minor fraction of a complex mixture, such as a portion of nucleic acid sequence due to a particular microorganism that constitutes only a very minor fraction of a particular biological sample. The nucleic acid or acids to be amplified may be obtained from plasmids such as pBR322, from cloned DNA or RNA, or from natural DNA or RNA from sources such as bacteria, yeast, viruses, and higher organisms such as plants or animals. DNA or RNA may be extracted from blood or tissue material such as chorionic villi or amniotic cells by a variety of techniques, including the well known technique of proteolysis and phenol extraction, as is common for preparation of nucleic acid for restriction enzyme digestion. In addition, suitable nucleic acid preparation techniques are described in Maniatis *et al.*, Molecular Cloning: A Laboratory Manual (New York, Cold Spring Harbor Laboratory, 1982), pp. 280-281; U.S. Patent Nos. 4,683,195 and 4,683,202; EP 258,017; and Saiki *et al.*, 1985, Biotechnology 3:1008-1012.

Any specific nucleic acid sequence can be produced by the PCR process. It is only necessary that a sufficient number of bases at both ends of the sequence be known in sufficient detail so that two oligonucleotide primers can be prepared which will hybridize to

different strands of the desired sequence at relative positions along the sequence such that an extension product synthesized from one primer, when it is separated from its template (complement), can serve as a template for extension of the other primer. The greater the knowledge about the bases at both ends of the sequence, the greater can be the specificity of the primers for the target nucleic acid sequence, and thus the greater the probability that the process will specifically amplify the target.

The specific amplified nucleic acid sequence produced by PCR is produced from a nucleic acid containing that sequence and called a template or "target." If the target nucleic acid contains two strands, the strands are separated before they are used as templates, either in a separate step or simultaneously with the synthesis of the primer extension products. This strand separation can be accomplished by any suitable denaturing method, including physical, chemical, or enzymatic means. One physical method of separating the nucleic acid strands involves heating the nucleic acid until it is completely (>99%) denatured. Typical heat denaturation involves temperatures ranging from about 80 to 105°C for times ranging from about 1 second to 10 minutes. Strand separation may also be induced by a helicase enzyme, or an enzyme capable of exhibiting helicase activity, e.g., the enzyme RecA, which has helicase activity and in the presence of riboATP is known to denature DNA. The reaction conditions suitable for separating the strands of nucleic acids with helicases are described in Cold Spring Harbor Symposia on Quantitative Biology, Vol. XLIII "DNA: Replication and Recombination" (New York, Cold Spring Harbor Laboratory, 1978), B. Kuhn *et al.*, "DNA Helicases", pp. 63-67, and techniques for using RecA are reviewed in Radding, 1982, *Ann. Rev. Genetics* 16:405-437.

When the complementary strands of the nucleic acid or acids are separated, whether the nucleic acid was originally double or single stranded, the strands are ready to be used as a template for the synthesis of additional nucleic acid strands. The amplification reaction is generally conducted in a buffered aqueous solution, preferably at a pH of 7 to 9 (all pH values herein are at room temperature) most preferably about pH 8. Preferably, a molar excess (for cloned nucleic acid, usually about 1000:1 primer:template, and for genomic nucleic acid, usually about 10⁶⁻⁸:1 primer:template) of the two oligonucleotide primers is added to the buffer containing the separated template strands. The amount of complementary strand may not be known, however, in many applications, so that the amount of primer relative to the amount of complementary strand may not be determinable with certainty.

The deoxyribonucleoside triphosphates dATP, dCTP, dGTP, and dTTP are also added to the PCR mixture in adequate amounts, and the resulting solution is heated to about 90-100°C for about 1 to 10 minutes, preferably from 1 to 4 minutes. If the target nucleic acid forms secondary structure, the nucleotide 7-deaza-2'-deoxyguanosine-5'-triphosphate is also employed, as is known in the art, to avoid the potential problems such secondary structure can cause. After heating, the solution is allowed to cool to room temperature, preferred for primer hybridization. To the cooled mixture is added a polymerization agent, and the polymerization reaction is conducted under conditions known in the art. This synthesis reaction may occur at temperatures primarily defined by the polymerization agent. Thus, for example, if an *E. coli* DNA polymerase is used as a polymerizing agent, the maximum temperature for polymerization is generally no greater than about 40°C. Most conveniently, the reaction using *E. coli* polymerase occurs at room temperature. For most PCR applications, however, the thermostable enzyme Taq polymerase is employed at much higher temperatures, typically 50 to 70°C.

Nevertheless, the polymerization agent for PCR may be any compound or system, including enzymes, which will function to accomplish the synthesis of primer extension products from nucleotide triphosphates. Suitable enzymes for this purpose include, for example, *E. coli* DNA polymerase I, Klenow fragment of *E. coli* DNA polymerase I, T4 DNA polymerase, other available DNA polymerases, reverse transcriptase (used in the first cycle of PCR if the target is RNA), and other enzymes, including heat-stable enzymes such as Taq polymerase, which will facilitate combination of the nucleotides in the proper manner to form the primer extension products which are complementary to a target nucleic acid strand. Generally, synthesis will be initiated at the 3' end of each primer and proceed in the 5' direction along the template strand, until synthesis terminates. There may be agents, however, which initiate synthesis at the 5' end and proceed in the other direction, and there seems no reason such agents could not also be used as polymerization agents in PCR.

The newly synthesized strand in PCR is base paired to a complementary nucleic acid strand to form a double-stranded molecule, which in turn is used in the succeeding steps of the PCR process. In the next step, the strands of the double-stranded molecule are separated to provide single-stranded molecules on which new nucleic acid is synthesized. Additional polymerization agent, nucleotides, and primers may be added if necessary for the reaction to proceed. The PCR steps of strand separation and extension product

synthesis can be repeated as often as needed to produce the desired quantity of the specific nucleic acid sequence.

As noted above, PCR has revolutionized the nucleic acid based diagnostics industry. European Patent Office Publication 237,362, incorporated herein by reference, discloses assay methods employing PCR. In EP 237,362, PCR-amplified DNA is fixed to a filter and then treated with a prehybridization solution containing SDS, Ficoll, serum albumin, and various salts. A specific oligonucleotide probe (of e.g., 16 to 19 nucleotides) is then added and allowed to hybridize. Preferably, the probe is labeled to allow for detection of hybridized probes. EP 237,362 also describes a "reverse" dot blot, in which the probe, instead of the amplified DNA, is fixed to the membrane.

The recent advent of PCR technology has enabled the detection of specific DNA sequences initially present in only minute (<1 ng) quantities. For example, Higuchi *et al.*, 1988, *Nature* 332:543-546, describe the characterization of genetic variation between individuals based on samples containing only a single hair. DNA was isolated from the hair by digestion and extraction and then treated under PCR conditions to obtain amplification. Specific nucleotide variations were then detected by either fragment length polymorphism (PCR-FLP), hybridization to sequence-specific oligonucleotide (SSO) probes (a technique also described in Saiki *et al.*, 1986, *Nature* 324:163-166) or by direct sequencing via the dideoxy method (using amplified DNA rather than cloned DNA). Because PCR results in the replication of a DNA sequence positioned between two primers, insertions and deletions between the primer sequences result in product sequences of different lengths, which can be detected by sizing the product in PCR-FLP. In SSO hybridization, the amplified DNA can be fixed to a nylon filter by UV irradiation in a series of "dot blots" and, in one variation of the technique, then allowed to hybridize with an oligonucleotide probe labeled with HRP under stringent conditions. After excess probe is removed by washing, 3, 3', 5, 5'-tetramethylbenzidine (TMB) and H₂O₂ are added: HRP catalyzes H₂O₂ oxidation of TMB to a blue precipitate, the presence of which indicates hybridized probe. U.S. Patent No. 4,789,630, incorporated herein by reference, describes protocols and TMB compounds useful for purposes of the present invention. One may alternatively use one of the other leuco dyes (such as a red leuco dye developed by DuPont and licensed to Kodak) to indicate the presence of HRP. However, any chromogen that develops precipitable color or fluorescence as a consequence of peroxidatic activity can be used to detect HRP-labeled reagents. In fact, any enzyme can be used to label, so long as

there exists a colorless substrate which forms a colored or fluorescent product as a result of enzyme activity and the product can be captured on a solid support. Separate dot blot hybridizations are performed for each allele tested.

Church *et al.*, 1984, Proc. Natl. Acad. Sci. USA 81:1991-1995, discloses a method for genomic sequencing which comprises cross-linking restriction enzyme-digested genomic DNA fragments to nylon membranes using UV irradiation, and probing the bound fragments with comparatively long (100-200 bp) DNA probes. Church *et al.* also discloses that NTPs dried onto nylon membranes and UV irradiated at 0.16 KJ/m² for two minutes are bound more stably (i.e., TTP = 130x, dGTP = 30x, dCTP = 20x, and dATP = 10x) than non-UV irradiated nucleotides. Primary amino groups are highly reactive with 254 nm light-activated thymine (see Saito *et al.*, 1981, Tetrahedron Lett. 22:3265-68), and this reactivity is believed to be the mechanism by which nucleotides become covalently bound to a membrane.

The detection of genetic variations using SSO probes is typically performed by first denaturing and immobilizing the sample DNA on a nylon or nitrocellulose membrane. The membrane is then treated with short (15-20 base) oligonucleotides under stringent hybridization conditions, allowing annealing only in cases of exact complementarity. A large number of hybridizations must be performed when a sample is examined for the presence of many different sequences. For example, a test for the most common genetic mutations that lead to beta-thalassemia in Mediterranean populations would involve 12 probes and require 12 separate hybridizations, accomplished either by probing one filter 12 times or by conducting simultaneous hybridizations on 12 replicate filters (or some combination thereof). A DNA-based HLA typing test can require 20 to 50 probes and hybridizations, a prohibitive effort if one uses the prior art methods that require either splitting the sample into as many portions as there are probes or blotting the sample followed by probing with a single probe and then removing the probe, a process that must be repeated for each probe tested.

In traditional nucleic acid detection by oligomer hybridization, the DNA in the test sample, including the hybridization target, is noncovalently chemisorbed onto a solid support such as nitrocellulose or nylon and then hybridized to a labeled target-specific probe which, except in the SSO methodology just described, usually contains hundreds to thousands of nucleotides and is made biosynthetically. This method suffers from multiple deficiencies. The noncovalent target capture generally is weak enough that considerable

target may be washed from the solid support during detection (see, for example, Gingeras *et al.*, 1987, Nucleic Acids Research 15:5373-5390 and Gamper *et al.*, 1986, Nucleic Acids Research 14:9943-9954). Target chemisorption reduces the reactivity of the target sequence toward hybridization with probe. The capture and hybridization processes normally take many hours to reach completion. The need to chemisorb the target immediately before detection prevents the manufacture of a storage-stable capture reagent with built-in target specificity that can be applied rapidly to test samples. The sequence non-specificity of capture complicates the examination of a single test sample with more than one probe: either a lot of test sample must be available to load different solid supports to incubate with the various probes or a lot of time must be consumed in serial probing of a singly immobilized test sample.

Ranki *et al.*, 1983, Gene 21:77-85, improve on the traditional technology by creating a sequence-specific capture reagent, capturing the target sequence from the test sample by nucleic acid hybridization and increasing specificity by detecting captured target with a second, labeled, sequence-specific nucleic acid probe. However, their technology still suffers from multiple deficits. The sequence-specific capture probe is immobilized by chemisorption, so that the assay still is vulnerable to signal attenuation by desorption of both capture probe and probe-target complex during the incubations and washes. Chemisorption reduces probe reactivity, requiring long incubation times to maximize capture efficiency. Two nucleic acid probes must be manufactured instead of one. The capture and detection probes of Ranki *et al.* are so large that they must be prepared by biosynthetic instead of much cheaper chemical synthetic routes. A small capture probe would not be immobilized efficiently by chemisorption.

Gingeras *et al.*, 1987, *supra*, improve further on DNA probe technology by covalently attaching relatively short, chemically synthesized, oligonucleotide hybridization probes to a solid support, dramatically reducing hybridization time. However, direct coupling of the target-specific sequence to the support risks reduced reactivity caused by steric occlusion by the support. Furthermore, the method demonstrated no way of detecting captured DNA apart from the incorporation of radioactive label into the target, a procedure which is relatively hazardous and inconvenient. Finally, the beaded solid support of Gingeras *et al.* is hard to adapt to assays in which multiple targets are probed, because the test sample must be exposed to separate containers of beads carrying the different probes, taking care not to mix beads with different specifications.

Gamper *et al.*, *supra*, describe a different strategy to accelerate oligomer hybridization: oligo hybridization is performed in solution rather than on a solid support, the hybrid species being simultaneously photochemically trapped, because the target-specific oligomer has been chemically modified with a moiety which crosslinks double-stranded DNA when irradiated. However, apart from the expense of creating the photo-adduct labeling reagent, this method suffers the inconvenience and delay associated with ultrafiltration to remove the considerable excess of unreacted probe, followed by gel electrophoresis to purify the hybridization product to the point that it can be identified. This procedure would be particularly inconvenient to adapt to simultaneous probing of multiple targets, because of the need to engineer targets to be electrophoretically resolvable.

The present invention provides a particularly advantageous assay method which permits the simultaneous nonisotopic detection of two or more specific nucleic acid sequences or control conditions in a single test sample, using a single solid support divided into discrete regions to which different oligonucleotide probes have been covalently attached via spacer arms. The method comprises:

- (a) attaching the probes to defined regions of the solid support through spacer arms, attached at one end to the probe and at the other end to the support;
- (b) reacting the test sample with the probe-bearing solid support under conditions promoting hybridization of the probes to any single-stranded complementary nucleic acid sequences in the test sample;
- (c) washing away any nucleic acid not hybridized to probe; and
- (d) detecting the probe-captured nucleic acid, preferably nonisotopically.

Because of the permanence of covalent attachment, the preparation of immobilized probes can be separated in time from their use, permitting manufacture of a storage-stable detection reagent, the hybridization capture support, which can be used rapidly to detect target nucleic acid sequences in test samples on demand. Covalent probe attachment and the use of a spacer arm between support and probe greatly accelerate and improve the efficiency of hybridization. The use of a dimensionally stable solid support with discrete regions for different probes greatly improves the economics, simplifies the physical format, and increases the reliability of hybridization and detection, because all target sequences in a simple test sample and all control conditions can be probed simultaneously in a single short

incubation and because all probe-target hybrids are exposed to identical incubation, wash, and detection conditions. Nonisotopic detection, whether via colored or fluorescent labels directly attached to the target nucleic acid or via colored, fluorescent, or enzyme labels indirectly attached to the target nucleic acid through a specific binding reaction, is much safer and more convenient than detection of radioactive atoms attached to the target nucleic acid, especially when developing storage-stable detection reagents and assay kits.

5 The invention also relates to a novel, stable, assay reagent comprising oligonucleotide probes covalently attached to discrete regions of a solid support via spacer arms, which probes have sequences designed to hybridize to different analyte nucleic acids in the test sample or to indicate different (positive or negative) control conditions which test the validity of the assay conditions. This assay reagent will have significant commercial
10 impact, being ideally suited to large-scale, automated, manufacturing processes and having a long shelf life. The reagent will prove especially useful in situations where the number of target sequences exceeds the number of samples tested. In general, the greater the number of target sequences and therefore immobilized probes, the greater the improvement of the invention over what has gone before. With PCR-amplified DNA samples, a simple test can easily be assayed for over fifty specific sequences on a single solid support. The nonisotopic detection aspect of the invention is especially well suited to target sequences generated by PCR, a process which permits covalent attachment to all target molecules of colored or fluorescent dyes and of binding moieties like biotin, of colored or fluorescent
20 and of binding moieties like biotin, digoxin, and specific nucleic acid sequences. The methods and reagents of the invention are also suited to detection of isotopically labeled nucleic acid, although this mode is not preferred.

An important aspect of the invention relates to a specific chemistry for attaching oligonucleotide probes to a solid support in a way which is especially suitable to large-scale
25 manufacture and which permits maximization of probe retention and hybridization efficiency. This chemistry comprises covalent attachment of a polynucleotide (preferably poly-dT) tail to the probe and fixation by the ultraviolet irradiation of the photoreactive tailed probe to a solid support bearing primary or secondary amines (e.g., a nylon membrane). However, the invention provides numerous alternative, non-photochemical
30 ways to attach probe to support, wherein electrophilic reagents are used to couple the probe to the spacer and the spacer to the solid support in either reaction order, and wherein the spacer can be any of a large variety of organic polymers or long-chain compounds.

Another aspect of the invention relates to a DNA sequence detection kit, which kit comprises the stable assay reagent, essentially a solid support having oligonucleotide probes covalently bound thereto via a spacer arm. The kit can also include PCR reagents, including PCR primers selected for amplification of DNA sequences capable of hybridizing with the oligonucleotide probes.

5 To aid in understanding and describing the invention, the following terms are defined below:

"Allele-specific oligonucleotide" (ASO) refers to a probe that can be used to distinguish a given allelic variant from all other allelic variants of a particular allele by hybridization under sequence-specific hybridization conditions.

10 "DNA polymorphism" refers to the condition in which two or more different variations of a nucleotide sequence exist in the same interbreeding population.

"Genetic disease" refers to specific deletions and/or mutations in the genomic DNA of an organism that are associated with a disease state and include sickle cell anemia, cystic fibrosis, alpha-thalassemia, beta-thalassemia, and the like.

15 "Label" refers to any atom or molecule which can be used to provide a detectable (preferably quantifiable) signal and which can be attached to a nucleic acid or protein. Labels may provide signals detectable by fluorescence, radioactivity, colorimetry, X-ray diffraction or absorption, magnetism, enzymatic activity, and the like. Suitable labels include fluorophores, chromophores, radioactive atoms (particularly ^{32}P and ^{125}I), electron-
20 dense reagents, enzymes, and ligands having specific binding partners. Enzymes are typically detected by their activity. For example, HRP can be detected by its ability to convert diaminobenzidine (more preferably, however, TMB is used) to a blue pigment, quantifiable with a spectrophotometer. It should be understood that the above description is not meant to categorize the various labels into distinct classes, as the same label may
25 serve in several different modes. For example, ^{125}I may serve as a radioactive label or as an electron-dense reagent. HRP may serve as enzyme or as antigen for an antibody, such as a monoclonal antibody (MAb). Further, one may combine various labels for desired effect. For example, MABs and avidin can be labeled and used in the practice of this invention. One might label a probe with biotin, and detect its presence with avidin labeled
30 with ^{125}I or with an antibiotin MAb labeled with HRP. Alternatively, one may employ a labeled MAb to dsDNA (or hybridized RNA) and thus directly detect the presence of hybridization without labeling the nucleic acids. Other permutations and possibilities will

be readily apparent to those of ordinary skill in the art and are considered within the scope of the instant invention.

"Oligonucleotide" refers to primers, probes, oligomer fragments, oligomer controls, and unlabeled blocking oligomers and is a molecule comprised of at least two or more deoxyribonucleotides or ribonucleotides. An oligonucleotide can also contain
5 nucleotide analogues, such as phosphorothioates and alkyl phosphonates, and derivatized (i.e., labeled) nucleotides. The exact size of an oligonucleotide will depend on many factors, which in turn depend on the ultimate function or use of the oligonucleotide.

"Primer" refers to an oligonucleotide, whether occurring naturally or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed
10 under conditions in which synthesis of a primer extension product complementary to a nucleic acid strand can occur. The primer is preferably an oligodeoxyribonucleotide and is single stranded for maximum efficiency in amplification, but may alternatively be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. The primer must be sufficiently long to prime the
15 synthesis of extension products in the presence of the polymerase, but the exact length of a primer will depend on many factors. For example, for diagnostics applications, the oligonucleotide primer typically contains 15 to 25 nucleotides. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with template. Suitable primers for amplification are prepared by means known to those of
20 ordinary skill in the art, for example by cloning and restriction of appropriate sequences, direct chemical synthesis, and purchase from a commercial supplier. Chemical methods for primer synthesis include: the phosphotriester method described in Narang *et al.*, 1979, *Meth. Enzymol.* 68:90 and U.S. Patent No. 4,356,270; the phosphodiester method disclosed in Brown *et al.*, 1979, *Meth. Enzymol.* 68:109; the diethylphosphoramidite
25 method disclosed in Beaucage *et al.*, 1981, *Tetrahedron. Lett.* 22:1859-1862; and the solid support method disclosed in U.S. Patent No. 4,458,066. The primers may also be labeled, if desired.

"Restriction fragment length polymorphism" (RFLP) refers to a DNA
polymorphism at a restriction enzyme recognition site. The restriction enzyme specific for
30 the polymorphic site can be used to digest sample DNA, and when the digested DNA is fractionated by electrophoresis and, if necessary, treated for visualization, different samples

produce different restriction endonuclease patterns, depending on the particular polymorphic sequence present in the sample.

"Sequence-specific hybridization" refers to strict hybridization conditions in which exact complementarity between probe and sample target sequence is required for hybridization to occur. Such conditions are readily discernible by those of ordinary skill in the art and depend upon the length and base composition of the probe. In general, one may vary the temperature, pH, ionic strength, and concentration of chaotropic agent(s) in the hybridization solution to obtain conditions under which substantially no probes will hybridize in the absence of an "exact match." For hybridization of probes to bound DNA, the empirical formula for estimating optimum temperature under standard conditions (0.9 M NaCl) is: $T(^{\circ}\text{C}) = 4(N_G + N_C) + 2(N_A + N_T) - 5^{\circ}\text{C}$, where N_G , N_C , N_T , and N_A are the numbers of G, C, A, and T bases in the probe (J. Meinkoth et al., 1984, Anal. Biochem. 138:267-284). Those of skill in the art recognize, however, that this calculation only gives an approximate value for optimum temperature, which should then be empirically tested to obtain the true optimum temperature. The probe utilized in a sequence-specific hybridization is called a "sequence-specific oligonucleotide" (SSO), which can also be an allele-specific oligonucleotide (ASO). Those of skill in the art recognize that for a single mismatch between probe and target to be destabilizing, the hybridizing region of the probe must be relatively short, generally no longer than about 23 bases, and usually about 17 to 23 bases in length.

"Specific binding partner" refers to a protein capable of binding a ligand molecule with high specificity, as for example in the case of an antigen and an antibody or MAb specific therefor. Other specific binding partners include biotin and avidin, streptavidin, or an anti-biotin antibody; IgG and protein A; and the numerous receptor-ligand couples known in the art.

To aid in understanding the invention, several Figures accompany the description of the invention. These Figures are briefly described below.

Figure 1 depicts a plot of probe binding as a function of UV exposure.

Figure 2 depicts a plot of hybridization efficiency as a function of UV exposure.

Figure 3 depicts a series of dot blots demonstrating the presence of either normal beta-globin or sickle cell beta-globin, obtained by sandwich assay.

Figure 4 depicts a series of dot blots demonstrating the presence of either normal beta-globin or sickle cell beta-globin, obtained by direct assay.

Figure 5 depicts a series of dot blots demonstrating HLA DQalpha genotyping.

Figure 6 depicts a series of dot blots demonstrating beta-thalassemia typing.

The present invention provides a method for detecting the presence of a specific nucleotide sequence in a sample by contacting the sample with immobilized oligonucleotide probes under conditions that allow for hybridization of complementary nucleic acid sequences. In one embodiment of the method, the test sample is contacted with a solid support upon which are immobilized probes specific for one or more target sequences (the "analyte"), probes for a positive control sequence that should be present in all test samples, and optionally probes for a negative control sequence that should not be present in any test sample — each different probe is immobilized at a distinct region on the solid support. If an analytical signal is detected in the negative control region or if no analytical signal is detected in the positive control region, then the validity of the response in the analyte region is suspect, and the sample should be retested. In another preferred embodiment, a number of different analyte-specific probes are covalently attached to distinct regions of a solid support so that one can test for allelic variants of a given genetic locus in a single hybridization reaction. In still another preferred embodiment, the various analyte-specific probes are complementary to nucleotide sequences present in various microorganisms.

The immobilized probes are also an important aspect of the invention. The probes comprise two parts: a hybridizing region composed of a nucleotide sequence of about 10 to 50 nucleotides (nt) and a spacer arm, at least as long as the hybridizing region, which is covalently attached to the solid support and which acts as a "spacer", allowing the hybridizing region of the probe to move away from the solid support, thereby improving the hybridization efficiency of the probe. In a preferred embodiment, the spacer arm is a sequence of nucleotides, called the "tail", that serves to anchor the probes to the solid support via covalent bonds between nucleotides within the tail of the probe and reactive groups within the solid support matrix.

As described more fully below, the immobilized probes of the invention avoid the problems inherent in prior art detection methods with immobilized probes. These problems include a lack of sensitivity, for many prior art methods for immobilizing probes actually result in the hybridizing region of the probe becoming attached to the solid support and thus less free to hybridize to complementary sequences in the sample. In addition, the prior art methods for synthesizing and attaching the spacer to the probe and to the solid support are complicated, time-consuming, expensive, and often involve the use of toxic reagents. In

marked contrast, a preferred method of the invention for synthesizing and immobilizing probes is quickly completed with readily available, relatively nontoxic, reagents, and with practically no chemical manipulations. The polynucleotide tails of the invention are composed of nucleotides that are attached to the hybridizing region with an enzyme or with the aid of commercially available nucleic acid synthesizers. The tails of the invention are attached to the solid support by a similarly problem-free method: exposure to ultraviolet (UV) light.

Those of skill in the art recognize that nucleic acid hybridization serves as the basis for a number of important techniques in the medical diagnostics and forensics industries. In addition, nucleic acid hybridization serves as an important tool in the laboratories where scientific advances in many diverse fields occur. The present invention represents an important step in making nucleic acid based diagnostics even more powerful and useful. As noted above, PCR has played an important role in these same industries and laboratories, and the present invention will often be practiced on samples in which the nucleic acid has been amplified by PCR. Various useful embodiments of the present invention are described below, but the full scope of the invention can only be realized when understood and utilized by the various and diverse practitioners of nucleic acid based diagnostics.

One very important use of the present invention relates to the detection and characterization of specific nucleic acid sequences associated with infectious diseases, genetic disorders, and cellular disorders, including cancer. In these embodiments of the invention, amplification of the target sequence is again useful, especially when the amount of nucleic acid available for analysis is very small, as, for example, in the prenatal diagnosis of sickle cell anemia using DNA obtained from fetal cells. Amplification is particularly useful if such an analysis is to be done on a small sample using non-radioactive detection techniques which may be inherently insensitive, or where radioactive techniques are being employed but where rapid detection is desirable.

The immobilized probes provided by the present invention not only are useful for detecting infectious diseases and pathological abnormalities but also are useful in detecting DNA polymorphisms not necessarily associated with a pathological state. The term forensic is most often used in a context pertaining to legal argument or debate. Individual identification on the basis of DNA type is playing an ever more important role in the law. For example, DNA typing can be used in the identification of biological fathers and so

5 serves as an important tool for paternity testing. DNA typing can also be used to match biological evidence left at the scene of a crime with biological samples obtained from an individual suspected of committing the crime. In a similar fashion, DNA typing can be used to identify biological remains, whether those remains are a result of a crime or some non-criminal activity. The practice of forensic medicine now routinely involves the use of DNA probes, in protocols that can be made more efficient by employing the present invention.

10 To achieve the important and diverse benefits of the present invention, one must first synthesize the probes to be immobilized on a solid support. The probe sequence can be synthesized in the same manner as any oligonucleotide, and a variety of suitable synthetic methods were noted above in the discussion of PCR primers. The hybridizing region of the probes of the invention is typically about 10 to 50 nt in length, and more often 17 to 23 nt in length, but the exact length of the hybridizing region will of course depend on the purpose for which the probe is used. Often, for reasons apparent to those of skill in the art, the hybridizing region of the probe will be designed to possess exact
15 complementarity with the target sequence to be detected, but once again, the degree of complementarity between probe and target is somewhat tangential to the present invention. The probes of the invention are, however, preferably "tailed" with an oligonucleotide sequence that plays a critical role in obtaining the benefits provided by the present invention.

20 This tail of the probes of the invention consists of ribonucleotides or deoxyribonucleotides (e.g., dT, dC, dG, and dA). The nucleotides of the tail can be attached to the hybridizing region of the probe with terminal deoxynucleotidyl transferase (TdT) by standard methods. In addition, the entire tailed probe can be synthesized by chemical methods, most conveniently by using a commercially available nucleic acid synthesizer. One can also synthesize the tails and hybridizing regions separately and then
25 combine the two components. For instance, a preparation of tails can be prepared (and even attached to a solid support, such as a bead) and then attached to a preparation of hybridizing regions.

30 When using a DNA synthesizer to make the tailed probes of the invention, one should take steps to avoid making a significant percentage of molecules that, due to a premature chain termination event, do not contain a hybridizing region. One such step involves synthesizing the hybridizing region of the probe first, creating a tailed probe with

the hybridizing region at the 3' end of the molecule. Because the likelihood of a premature chain termination event increases with the length of the molecule, this step increases the likelihood that if a chain termination event occurs, the occurrence merely results in a shorter tail. However, because most premature chain termination events are a result of a failure to "de-block" during synthesis, and because the exact number of residues in the tail of a probe
5 of the invention is not critical, one may also merely omit the blocking and de-blocking steps during automated synthesis of the tail region of the probe. If these steps are omitted (during tail synthesis only), the tail can be placed on either the 5' or 3' end of the probe with equal efficiency and satisfactory results.

As noted above, the preferred spacer arms of the probes of the invention are
10 comprised of nucleotide tails. Because the tails serve to attach the probe to the solid support, the relative efficiency with which a given oligonucleotide will react with a solid support is important in choosing the sequence to serve as the tail in the probes of the invention. Most often, the tail will be a homopolymer, and Figure 1, below, depicts the relative efficiencies with which synthetic oligonucleotides with varying length
15 homopolymer tails were covalently bound to a nylon filter as a function of UV exposure. As shown in the figure, oligonucleotides with longer poly-dT tails were more readily fixed to the membrane, and all poly-dT tailed oligonucleotides attained their maximum values by 240 mJ/cm² of irradiation at 254 nm. In contrast, the poly-dC (400 nt in length) tail required more irradiation to crosslink to the membrane and was not comparable to the
20 equivalent poly-dT tail even after 600 mJ/cm² exposure. Untailed oligonucleotides were retained by the filter in a manner roughly parallel to that of the poly-dC-tailed probes.

Thus, the probes of the invention preferably comprise a poly-dT tail of greater than 10 thymidine (T) residues. Usually, the tail will comprise at least 100 T residues, and most preferably, the tail will comprise at least 400 dT nucleotides. As the poly-dT tail functions
25 primarily to bind the probe to the solid support, the exact number of dT nucleotides is not critical, as noted above. Although those of skill in the art will readily recognize the fact, it should be noted that the composition of the tail need not be homogeneous, i.e., a mixture of nucleotides may be used. Preferably, however, the tail will include a significant number of thymine bases, as T reacts most readily with the solid support by the preferred methods
30 for making the immobilized probes of the invention, which methods are discussed more fully below. If one desires to utilize the probes in sequence-specific hybridizations, one must be aware of the problem caused by creation of a random sequence in the

heterogeneous tail that closely resembles the hybridizing region of a probe. If a heterogeneous tail is employed, it is still desirable to maintain a distribution of 150 pyrimidine residues per tail, if the probes are to be fixed to a solid support by UV irradiation.

5 The tail should always be larger than the hybridizing region, and the greater the disparity in size between the hybridizing region and the tail (so long as the tail is larger), the more likely it is that the tail, rather than the hybridizing region, will react with the support, a preferred condition. Larger tails thus increase the likelihood that only the tail will participate in reacting with and thereby binding to the solid support. Because the tail also functions as a "spacer," enabling the complementary sequence to diffuse away from the
10 solid support where it may hybridize more easily, free of steric interactions, larger tails are doubly preferred. Excessively extended tails, however, are uneconomical, and, if carried to an extreme, excessive tailing could have adverse effects.

A preferred method of synthesizing a probe of the invention is as follows. The probe is synthesized on a DNA synthesizer (the Model 8700, marketed by Biosearch, is
15 suitable for this purpose) with beta-cyanoethyl N, N-diisopropyl phosphoramidite nucleosides (available from American Bionetics) using the protocols provided by the manufacturer. If desired, however, only the hybridizing region of the probe is synthesized on the instrument, and then 200 pmol of the probe are tailed in 100 μ l of reaction buffer at pH = 7.6 and containing 100 mM cacodylate, 25 mM Tris-base, 1 mM CoCl_2 , and 0.2 mM
20 dithiothreitol with 5 to 160 nmol deoxyribonucleotide triphosphate (dTTP) and 60 units (50 pmol) of terminal deoxyribonucleotidyl transferase (available from Ratliff Biochemicals) for 60 minutes at 37 degrees C (see Roydhouhury *et al.*, 1980, *Meth. Enz.* 65:43-62, for buffer preparation). Reactions are conveniently stopped by the addition of 100 μ l of 10 mM EDTA. The lengths of tails can be controlled by limiting the amount of dTTP (or other
25 nucleotide) present in the reaction mixture. For example, a nominal tail length of 400 dT residues is obtained by using 80 nmol of dTTP in the protocol described above.

Once the tailed probe of the invention is synthesized, the probe is then attached to a solid support. Suitable solid supports for purposes of the present invention will contain (or can be treated to contain) free reactive primary or secondary amino groups capable of
30 binding a UV-activated pyrimidine, especially thymine. Secondary amino groups may be preferred for purposes of the present invention. There are many ways to assure that a solid support (not necessarily nylon) has free, particularly secondary, amino groups.

Amine-bearing solid supports suitable for purposes of the present invention include polyethylenimine (chemisorbed to any solid, such as cellulose or silica with or without glutaraldehyde crosslinking) and silica or alumina or glass silanized with amine-bearing reagents such as PCR Inc.'s Prosil™ 220, 221, 3128, and 3202 reagents. Manville sells controlled porosity glass papers (Biomat™) appropriate for aminoalkyl silanization. One
5 may alkylate immobilized primary amines (e.g. with a methyl halide or with formaldehyde plus cyanoborohydride, (as described by Jentoff *et al.*, 1979, *J. Biol. Chem.* 254:4359-4365). As noted above, one may use a solid support to which polyethylenimine has been chemisorbed. Polyvinyl chloride sheets containing PEI-loaded silica are commercially available (manufactured by Amerace and sold by ICN as Protrans™ and by Polysciences
10 as Poly/Sep™), and PEI loading of cellulose is well known.

The solid support, also called a substrate, can be provided in a variety of forms, including membranes, rods, tubes, wells, dipsticks, beads, ELISA-format plates, and the like. A preferred support material is nylon, which contains reactive primary amino groups and will react with pyrimidines irradiated with UV light. Preferred solid supports include
15 charge modified nylons, such as the Genetrans-45™ membrane marketed by Plasco and the ZetaProbe™ membrane marketed by Bio-Rad.

Having chosen a suitable solid support, one makes the preferred immobilized probes of the invention by reacting a tailed oligonucleotide probe with the solid support under conditions that favor covalent attachment of the tail to the solid support. In a
20 preferred embodiment, the solid support is a membrane, and probe binding results from exposure of the probe on the membrane to UV irradiation, which activates the nucleotides in the tail, and the activated nucleotides react with free amino groups within the membrane. Careful dessication of tailed probes of the invention spotted onto a suitable solid support can also be used to facilitate covalent attachment of the probes to the substrate. One can
25 assay for the presence or absence in a solid support matrix of reactive groups capable of reacting with oligonucleotides by the procedure described in Example 1.

As is apparent from the foregoing, a preferred method for preparing the immobilized probes of the invention comprises fixing an oligonucleotide probe with a poly-
30 dT tail to a nylon membrane by UV irradiation. Although poly dT tails react very efficiently to solid supports by the methods of the present invention, efficiency of reaction of oligonucleotides with a membrane does not necessarily correlate with hybridization efficiency. One may therefore wish to determine the hybridization efficiency of a given

oligonucleotide probe after immobilization on a solid support. When the hybridization of various tailed probes is measured as a function of UV dosage, as shown in Figure 2, one observes that the optimum exposure changes with length of a poly-dT tail. Optimal exposures are about 20 mJ/cm² for 800 nt poly-dT tails and about 40 mJ/cm² for 400 nt poly-dT tails.

5 At 60 mJ/cm² exposure, one observes that oligonucleotides with longer tails hybridize more efficiently than can be accounted for by the additional amounts of probe reacted with and bound to the filter. This increased efficiency is believed to be due to a spacing effect: increasing the distance between the membrane and the hybridizing region of the immobilized probe may increase hybridization efficiency of the probe. Thus, too much
10 UV exposure during immobilization can not only damage the nucleotides in the probe but also can reduce the average spacer length and decrease hybridization efficiency. It is important to note that because dC tails react less efficiently (as compared to dT tails) with a membrane, hybridization efficiency of a poly-dC tailed probe reaches a plateau where loss due to UV damage and tail shortening is compensated for by the fixing of new molecules
15 to the membrane (see Figures 1 and 2). This characteristic of poly-dC tails may make such tails preferred when UV exposure cannot be carefully controlled.

 No matter what the base content of the tail of the probe, one may automate the attachment of probe to support in accordance with the method of the present invention. One semi-automated means of attachment preferred for positive charge nylon membranes is
20 as follows. A commercially available "dot-blot" apparatus can be readily modified to fit into a Perkin-Elmer/Cetus Pro/Pette® automated pipetting station; the membrane is then placed on top of the dot-blot apparatus and vacuum applied. The membrane dimples under the vacuum so that a small volume (5 to 20 µl) of probe applied forms a consistent dot with edges defined by the diameter of the dimple. No disassembly of the apparatus is required
25 to place and replace the membrane -- the vacuum may be kept constant while membranes are applied, spotted with probe, and removed.

 Once the probes are spotted onto the membrane, the spotted membrane is treated to immobilize the probes. A preferred method for covalently attaching the probes to a nylon membrane is as follows. Tailed oligonucleotides in TE buffer (10 mM Tris-HCl, pH =
30 8.0, and 0.1 mM EDTA) are applied to a Genetrans-45™ (Plasco) membrane with a BioDot™ (Bio-Rad) spotting manifold. The damp membranes, also called "filters," are then placed on paper pads soaked with TE buffer; the pads and filters are then placed in a

UV light box (the Stratalinker 1800TM light box marketed by Stratagene is suitable for this purpose) and irradiated at 254 nm under controlled exposure levels. UV dosage can be controlled by time of exposure to a particular UV light source or, more preferably, by measuring the radiant UV energy with a metering unit. Exposure time typically ranges from about 0.1 to 10 minutes, most often about 2 to 3 minutes. The support is preferably damp during irradiation, but if the support is dried first, a shorter UV irradiation exposure can be used. The irradiated filters are washed in a large volume of a solution composed of 5X SSPE (1X SSPE is 180 mM NaCl, 10 mM NaH₂PO₄, and 1 mM EDTA, pH = 7.2) and 0.5% sodium dodecyl sulfate (SDS) for about one-half hour at 55 degrees C to remove unreacted oligonucleotides. Filters can then be rinsed in water, air dried, and stored at room temperature until needed.

UV irradiation of nucleotides is known to cause pyrimidine photochemical dimerization, which, for purposes of the present invention, is not preferred. A number of steps can be taken to reduce dimerization during UV irradiation, including: applying the oligonucleotide probe to the membrane at a high pH, above 9 and preferably above 10; applying the probe to the membrane at a very low ionic strength, between 0 and 0.01; using the lowest probe concentration that gives the desired signal intensity; and irradiating with light excluding wavelengths longer than 250 nm, preferably with no light with a wavelength longer than 240 nm. However, the extent to which these steps could impair probe immobilization has not been tested. In general, spotting and attachment of the probe to the membrane should be done at a temperature and in a solvent that minimizes base-pairing and base-stacking in the probes.

After constructing the novel immobilized probes of the invention, one is ready to employ those probes in the useful nucleic acid sequence detection methods of the invention. In a preferred embodiment of this method, a sample suspected of containing a target nucleic acid sequence is treated under conditions suitable for amplifying the target sequence by PCR. Note that the process of "asymmetric" PCR, described by Gyllensten and Erlich, 1988, Proc. Natl. Acad. Sci. USA 85:7652-7656, for generation of single stranded DNA can also be used to amplify the sample nucleic acid. The PCR primers are biotinylated, for subsequent detection of hybridized primer-containing sequences. The amplification reaction mixture is denatured, unless asymmetric PCR was used to amplify, and then applied to a membrane of the present invention under conditions suitable for hybridization to occur (most often, sequence-specific hybridization). Hybridized probe is detected by

binding streptavidin-horseradish peroxidase (SA-HRP, available from a wide variety of chemical vendors) to the biotinylated DNA, followed by a simple colorimetric reaction in which a substrate such as TMB is employed. One can then determine whether a certain sequence is present in the sample merely by looking for the appearance of colored dots on the membrane.

5 In an especially preferred embodiment of the above method, a filter with immobilized oligonucleotides is placed in hybridization solution containing 5X SSPE, 0.5% SDS, and 100 ng/ml SA-HRP (as marketed under the SeeQuence™ by Cetus and Eastman Kodak). PCR-amplified sample DNA is denatured by heat or by addition of NaOH and EDTA and added immediately to the hybridization solution, which contains
10 enough SSPE to neutralize any NaOH present. The sample is then incubated at a suitable temperature for hybridization to occur (typically, as exemplified below, at 55 degrees C for 30 minutes). During this incubation, hybridization of product to immobilized oligonucleotide occurs as well as binding of SA-HRP to biotinylated product. The filters are briefly rinsed in 2X SSPE and 0.1% SDS at room temperature, then washed in the
15 same solution at 55-degrees C for 10 minutes, then quickly rinsed twice in 2X PBS (1X PBS is 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, and 8 mM Na₂HPO₄, pH = 7.4) at room temperature. Color development is performed by incubating the filters in red leuco dye or TMB at room temperature for 5 to 10 minutes. Photographs are taken of the filters after color development for permanent records.

20 Although the detection method described above is preferred, those of skill in the art recognize that the immobilized probes of the invention can be utilized in a variety of detection formats. One such format involves labeling the immobilized probe itself, instead of the sample nucleic acid. If the probe is labeled at or near the end of the hybridizing sequence (far from the site of attachment of the probe to the solid support), one can treat the
25 potentially hybridized sample DNA with an appropriate restriction enzyme, i.e., one that cleaves only duplex nucleic acids at a sequence present in the hybridizing region of the probe, so that restriction releases the label from the probe (and the membrane) for detection of hybridization. Suitable labels include peroxidase enzymes, acid phosphatase, radioactive atoms or molecules (e.g., ³²P, ¹²⁵I, etc.), fluorophores, dyes, biotin, ligands
30 for which specific monoclonal antibodies are available, and the like. If the primer or one or more of the dNTPs utilized in a PCR amplification has been labeled (for instance, the biotinylated dUTP derivatives described by Lo *et al.*, 1988, *Nuc. Acids Res.* 16:8719),

instead of the immobilized probe, then, as noted above, hybridization can be detected by assay for presence of label bound to the membrane.

The immobilized probes of the invention can also be used in detection formats in which neither probe nor primer is labeled. In such a format, hybridization can be detected using a labeled "second probe." The second probe is complementary to a sequence
5 occurring within the target DNA, but not overlapping the bound probe sequence; after hybridization of the second probe, the immobilized probe, second probe, and target sequence form a nucleic acid "sandwich," the presence of which is indicated by the presence of the label of the second probe on the membrane. One could also employ monoclonal antibodies (or other DNA binding proteins) capable of binding specifically to
10 duplex nucleic acids (e.g., dsDNA) in a detection format that uses no labeled nucleic acids. Those of skill in the art will recognize that one important advantage of the immobilized probes of the invention is the ability, at least with most detection formats, to recycle the support-bound probe by denaturing the hybridized complex, eluting the sample DNA, and treating the support (for example, by washing, bleaching, etc.) to remove any remaining
15 traces of extraneous DNA, label, developer solution, and immobilized dye (see U.S. Patent No. 4,789,630 and PCT application No. 88/0287, incorporated herein by reference).

Those of skill in the art will recognize the many and diverse uses for the immobilized probes of the present invention. One exciting application of these immobilized probes is in conjunction with the technique of simultaneous amplification of several DNA
20 sequences ("multiplex" PCR). Such simultaneous amplification can be used to type at many different loci with a single membrane. For instance, one can type for the polymorphic HindIII site in the γ gene (see Jeffreys, 1979, Cell 18:1-10), the polymorphic AvaII site in the low density lipoprotein receptor gene (see Hobbs *et al.*, 1987, Nuc. Acids Res. 15:379), and for polymorphisms in the HLA DQalpha gene
25 simultaneously by amplifying all three loci in a single PCR and applying the amplified material to a suitable set of immobilized probes of the present invention. Other genetic targets whose analysis would be simplified by this technique include the detection of somatic mutations in the ras genes, where six loci and 66 possible alleles occur (see Verlaan-de Vries *et al.*, 1986, Gene 50:313-320); the typing of DNA polymorphisms at the
30 HLA DP locus; the detection of beta-thalassemia in Middle Eastern populations, where in addition to the endogenous mutations, Mediterranean and Asian Indian mutations are

present at significant frequencies; the detection of infectious pathogens; and the detection of microorganisms in environmental surveys.

In many of these applications, it will be desirable to obtain a membrane on which are immobilized a diverse set of oligonucleotides specific for different sequences that nevertheless can hybridize under the same sequence-specific hybridization conditions. If
5 necessary, this situation can be achieved by adjusting the length, position, and strand-specificity of the probes, or by varying the amount of probe applied to the membrane, or by adding a salt, such as tetramethylammonium chloride, to the hybridization buffer to minimize differences among immobilized oligonucleotides caused by varying base
10 compositions (see Wood *et al.*, 1985, *Proc. Natl. Acad. Sci. USA* 82:1585-1588).

The examples below illustrate various useful embodiments of the invention and enable the skilled artisan to appreciate the invention more fully and so should not be construed as limiting the scope of the invention in any way.

Example 1

Probe Retention and Hybridization Efficiency

The stable binding of poly-dT-tailed probe sequences to nylon as a function of tail length and UV exposure was examined as described below. A 19-base oligonucleotide (RS18: 5'-CTCCTGAGGAGAAGTCTGC) was labeled at its 5' end with gamma ³²P-ATP and T4 polynucleotide kinase (see Saiki *et al.*, 1986, *Nature* 324:163-166). Portions
20 of the kinased probe were then tailed with dTTP and terminal transferase (TdT), as described by Roydhouhury *et al.* RS18 was present at a concentration of 2 μ M, TdT (Ratcliff Biochemicals, Los Alamos, NM) at 600 U/ml, and either dCTP or dTTP at either 0 μ M, 50 μ M, 100 μ M, 200 μ M, 400 μ M, or 800 μ M to prepare constructs with either dC or dT tails of approximately 0, 25, 50, 100, 200, 400 or 800 dT bases or 400 dC bases per
25 molecule. Reaction mixtures were incubated for 60 minutes at 37°C and were terminated by addition of an equal volume of 10 mM EDTA.

Four pmol of each sample diluted in 100 μ l of TE buffer were spotted onto nine duplicate filters (Genetrans-45 nylon, Plasco, Woburn, Mass.), UV irradiated for various times, washed to remove unbound oligonucleotides, and then each spot was measured by
30 scintillation counting to determine the amount of probe crosslinked to the nylon membrane. The values plotted in Figure 1 are relative to an unirradiated, unwashed control filter (100% retention). UV irradiation was accomplished by placing the filters in a Stratalinker 1800™

UV light box and irradiating the filters at 254 nm. Dosage was controlled using the internal metering unit of the device. The filters were then washed in 5X SSPE, 0.5% SDS for 30 minutes at 55°C to remove DNA not stably bound. The results plotted in Figure 1 show that even the non-tailed probe was retained by the membrane, but that retention of the untaild probe was not greatly improved by UV irradiation. The 400-dT tailed probe exhibited >90% retention after suitable exposure.

However, high retention does not necessarily correlate with high hybridization efficiency. Thus, hybridization efficiency was measured as follows. Probes were prepared with poly-dT tails as above, but with unlabeled RS18. The probes were spotted onto filters and UV irradiated, and excess probe was washed from the membrane by incubating the membrane in 5X SSPE, 0.5% SDS, for 30 minutes at 55°C. The membranes were then hybridized with 5 pmol of complementary ³²P-kinase labeled 40-mer (RS24: 5'-CCCACAGGGCAGTAACGGCAGACTTCTCCTCAGGAGTCAG, specific activity of 1.5 uCi/pmol) in a solution (10 ml) containing 5X SSPE and 0.5% SDS, at 55°C for 20 minutes, which are sequence-specific hybridization conditions. The membrane was then washed first with 2X SSPE, 0.1% SDS (3 x 100 ml) for 2 minutes at about 25°C, then in 2X SSPE, 0.1% SDS at 55°C for 5 minutes. The individual spots were excised, counted, and the counts plotted against UV exposure, as shown in Figure 2. The values plotted are fmol RS24 hybridized to the membrane. The results show that none of the non-tailed probe was able to hybridize under the conditions used, even though as much as 50% of the applied RS18 should be bound to the membrane. All of the tailed probes were able to hybridize, with hybridization efficiency increasing with increasing tail length. Optimal UV exposures were from about 60 to 120 mJ/cm².

Example 2

Sandwich Assay for Sickle-Cell Anemia

Two allele-specific probes were prepared, one for the normal beta-globin allele, called RS18, and one for the sickle-cell allele, RS21 (5'CTCCTGTGGAGAAGTCTGC); each probe had a 400 nt poly-dT tail. If desired, a probe for the hemoglobin C allele can be prepared with the sequence: 5'CTCCTAAGGAGAAGTCTGC. Eight replicate filters were prepared and spotted with 4, 2, 1, and 0.5 pmol of each tailed probe using the method set forth in Example 1, and then UV irradiated by placing the filters, DNA-side down, directly onto a TM-36 Transilluminator UV light box (U.V. Products, San Gabriel, CA)

for 5 minutes. Four 1 µg samples of genomic DNA (from cell lines Molt4 (beta^Abeta^A), SC-1 (beta^Sbeta^S), M + S (beta^Abeta^S), and GM2064 (beta^Abeta^A), a beta-globin deletion mutant) were subjected to 30 PCR amplification cycles with the primer pair PC03 (5'ACACAACCTGTGTTCACTAGC) and KM38 (5'TGGTCTCCTTAAACCTGTCTTG).

5 PCR was carried out in substantial accordance with the procedure described by Saiki *et al.*, 1988, *Science* 239:1350-1354. The DNA samples were amplified in 100 µl of reaction buffer containing 50 mM KCl, 10 mM Tris-HCl (pH = 8.4), 1.5 mM MgCl₂, 100 µg/ml gelatin, 200 µM each of dATP, dCTP, dGTP, and dTTP, 0.2 µM of each primer, and 2.5 units of Taq DNA polymerase (Perkin-Elmer/Cetus Instruments). The cycling reaction was performed on a programmable heat block, the DNA Thermal Cycler, available
10 from PECL, set to heat at 95 degrees C for 15 seconds (denature), cool at 55 degrees C for 15 seconds (anneal), and incubate at 72 degrees C for 30 seconds (extend) using the Step-Cycle program. After 30 cycles, the samples were incubated an additional 5 minutes at 72 degrees C.

15 Each amplification product (18 µl) was denatured by heating at 95°C for 5 minutes in 1 ml of TE, and then quenched on ice. A solution (4 ml) of 6.25X SSPE, 6.25X Denhardt's, and 0.625% SDS was mixed with 1 ml of each denatured PCR product, hybridized to one of the filters for 15 minutes at 55°C, washed with 2X SSPE, 0.1% SDS (3 X 100 ml) for 2 minutes at about 25°C, and then washed with 2X SSPE, 0.1% SDS (1 x 100 ml) for 5 minutes at 55°C.

20 The membranes were then equilibrated in 2X SSPE, 0.1% (v/v) Triton X-100 (100 ml) for 3 minutes at about 25°C to remove SDS. All of the filters were then hybridized in the same buffer with a horseradish peroxidase (HRP) labeled 15-mer, RS111 (5'-GCAGGTTGGTATCAA), specific for the PC03/KM38 amplification product, prepared by the method disclosed in PCT publications WO 89/02931 and 89/02932, incorporated herein
25 by reference.

30 These methods essentially involve derivatizing the nucleic acid probe using a linear linking molecule comprising a hydrophilic polymer chain (e.g., polyoxyethylene) having a phosphoramidite moiety at one end and a protected sulfhydryl moiety at the other end. The phosphoramidite moiety couples to the nucleic acid probe by reactions well known in the art (e.g., Beaucage *et al.*, 1981, *Tetrahedron Lett.* 22:1859-1862), while the deprotected sulfhydryl group can form disulfide or other covalent bonds with a protein, e.g., HRP. The HRP is conjugated to the linking molecule through an N-maleimido-6-aminocaproyl

group. The label is prepared by esterifying N-maleimido-6-aminocaproic acid with sodium 4-hydroxy-3-nitrobenzene sulfonate in the presence of one equivalent of dicyclohexylcarbodiimide in dimethylformamide. After purification, the product is added to phosphate buffer containing HRP at a weight ratio of 8:1 HRP to ester. The oligonucleotide probe is synthesized in a DNA synthesizer, and the linking molecule having the structure $(C_6H_5)_3CS-(CH_2CH_2O)_4-P(CH_2CH_2CN)[N(i-Pr)_2]$ is attached using phosphoramidite synthesis conditions. The trityl group is removed, and the HRP derivative and probe derivative are mixed together and allowed to react to form the labeled probe. A biotin-labeled probe may be prepared by similar methods.

The membrane was incubated with 4 pmol RS111 in a solution (8 ml) composed of 5X SSPE, 5 x Denhardt's, and 0.5% Triton X-100 for 10 minutes at 40°C. Following incubation, the membrane was washed with 2X SSPE, 0.1% Triton X-100 (3 x 100 ml) for 2 minutes at about 25°C.

The reaction was followed by color development (Sheldon *et al.*, 1986, *Proc. Natl. Acad. Sci. USA* 83:9085-9089) with TMB/H₂O₂, as shown in Figure 3. The membranes were soaked in 100 ml of color development buffer B (CDB-B: 237 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 8.0 mM Na₂HPO₄, pH 7.4, 5% (v/v) Triton X-100, 1 M urea, and 1% dextran sulfate), followed by washes with 2 x 100 ml of CDB-C (100 mM sodium citrate, pH 5.0) for 2 minutes at room temperature. Color was developed by replacing the CDB-C solution with 100 ml of CDB-D (100 mM sodium citrate, pH 5.0, 0.1 mg/ml 3,3',5,5'-tetramethylbenzidine), adding 50 µl of 3% H₂O₂, and allowing the color to develop for 30 minutes. The beta-globin genotypes of the amplified DNA samples were readily apparent from the filters, and good signal intensity was obtained even from the 0.5 pmol spot.

Example 3

Direct Assay for Sickle-Cell Anemia

A second set of DNAs (as described in Example 2 above) was amplified with PC03 and BW19. BW19 has the sequence 5'CAACTTCATCCACGTTTCACC, and is covalently bound to a molecule of biotin at the 5' end. Twelve µl of each of these amplification products were denatured as described in Example 2, added to 4 ml of hybridization buffer (6.25X SSPE, 6.25X Denhardt's, and 0.625% SDS), and incubated with the membrane-bound probe (the remaining four filters from Example 2 above) at 55°C for 15 minutes.

The membranes were then washed with 2X SSPE, 0.1% SDS (3 x 100 ml) for 3 minutes at room temperature, followed by a wash with 2X SSPE, 0.1% SDS (1 x 100 ml) for 5 minutes at 55°C.

5 The membranes were pooled together and equilibrated in 100 ml of CDB-A for 5 minutes at about 25°C (CDB-A: 237 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 8.0 mM Na₂HPO₄, pH 7.4, and 5% Triton X-100). The membranes were then placed in a heat-sealable bag with 10 ml of CDB-A and See-Quence™ SA-HRP conjugate (Cetus Corporation, Emeryville, CA) at 0.3 µg/ml and gently shaken for 10 minutes at about 25°C. Excess conjugate was removed by washing with CDB-A (3 x 100 ml for 3 minutes at 25°C), CDB-B (1 x 100 ml for 5 minutes at 25°C), and CDB-C (2 x 100 ml for 3
10 minutes at 25°C). The membranes were then equilibrated in CDB-D (100 ml) for 5 minutes at room temperature, followed by addition of 50 µl of 3% H₂O₂. The color was allowed to develop for 30 minutes with gentle shaking, followed by washing under deionized water for 10 minutes. The four filters after color-development are shown in Figure 4. The intensity and specificity of signals detected by this method compare favorably to those
15 obtained by sandwich hybridization. The faint signal in GM2064 was due to beta-globin contamination in that DNA sample.

Example 4

HLA DQalpha Genotyping

20 The DQalpha test is derived from a PCR-based oligonucleotide typing system that partitions the polymorphic variants at the DQalpha locus into four DNA major types denoted DQA1, DQA2, DQA3, and DQA4, three DQA4 subtypes, DQA4.1, DQA4.2, and DQA4.3, and three DQA1 subtypes, DQA1.1, DQA1.2, and DQA1.3 (see Higuchi *et al.*, 1988, *Nature* 332:543-546 and Saiki *et al.*, 1986, *Nature* 324:163-166).

25 Four oligonucleotides specific for the major types, four oligonucleotides that characterized the subtypes, and one control oligonucleotide that hybridizes to all allelic DQalpha sequences were given 400 nt poly-dT tails and spotted onto 12 duplicate nylon filters. About 2 to 10 pmol of each probe were placed in each spot.

30 With regard to amount of probe spotted, however, one may wish to employ lower amounts of RH54 and GH64, i.e., 0.035 pmol RH54 per spot is preferred. These probes are positive control probes for amplification and will hybridize to any DQalpha alleles under the conditions described. By reducing the amount of positive control probe on the

membrane, one can make the positive control probe the least sensitive probe on the membrane. Then, if insufficient amplified DQalpha DNA is applied to the membrane, one can recognize the problem, for the positive control probe will not react or will react only very weakly. Otherwise, when insufficient sample DNA is applied to the membrane, one runs the risk of misreading a heterozygous type as a homozygous type, because some probes hybridize less efficiently than others.

After spotting, the membranes were irradiated at 40 mJ/cm². The sequences of the hybridizing regions of the resulting immobilized probes are shown below.

| | <u>DQA Type</u> | <u>Designation</u> | <u>Sequence</u> |
|----|-------------------------|--------------------|---|
| 10 | A1 | GH75 RH83 | 5'-CTCAGGCCACCGCCAGGCA or 5'-GAGTTCAGCAAATTTGGAG |
| | A2 | RH71 RH82 | 5'-TTCCACAGACTTAGATTTG or 5'-TTCCACAGACTTAGATTTGAC |
| | A3 | GH67 | 5'-TTCCGCAGATTTAGAAGAT |
| | A4 | GH66 | 5'-TGTTTGCCTGTTCTCAGAC |
| 15 | A1.1 | GH88 | 5'-CGTAGAACTCCTCATCTCC |
| | A1.2, 1.3, 4 | GH89 | 5'-GATGAGCAGTTCTACGTGG |
| | A1.3 | GH77 | 5'-CTGGAGAAGAAGGAGAC |
| | not A1.3 | GH76 | 5'-GTCTCCTTCCTCTCCAG |
| 20 | all | RH54 | 5'-CTACGTGGACCTGGAGAG- GAAGGAGACTGCCTG or 5'-TGGACCTGGAGAGGAAGGAGACTG |
| | | GH64 | |
| | A4.2, A4.3, not A4.1 | HE01 | 5'-CATCGCTGTGACAAAACAT |

Although most of the probes are uniquely specific for one DQA type, two of the DQA1 subtyping probes cross hybridize to several DNA types. GH89 hybridizes to a sequence common to the DQA1.2, 1.3, and 4 types, and the probe GH76 detects all DQA types except DQA1.3. The GH76 probe is needed to distinguish DQA1.2/1.3 heterozygotes from DQA1.3/1.3 homozygotes. Further, the length and strand specificity of the probes were adjusted so that their relative hybridization efficiencies and stringency requirements for allelic discrimination were approximately the same. These eight probes produce a unique hybridization pattern for each of the 21 possible DQA diploid combinations.

The sequence variation that defines the DQalpha DNA types is localized within a relatively small hypervariable region of the second exon that can be encompassed within a single 242 bp PCR amplification product (see Horn *et al.*, 1988, Proc. Natl. Acad. Sci. USA 85:6012-6016). These primers are shown below.

Primer RS134 is 5'-GTGCTGCAGGTGTAAACTTGTAACAG

5

Primer RS135 is 5'-CACGGATCCGGTAGCAGCGGTAGAGTTG

Biotinylated PCR primers were used to amplify this 242 bp DQalpha sequence from several genomic DNA samples: six homozygous cell lines and six heterozygous individuals.

The biotinylated primers were synthesized as follows. Primary amino groups were introduced at the 5' termini of the primers by a variation of the protocols set forth in Coull *et al.*, 1986, Tetrahedron Lett. 27:3991-3994 and Connolly, 1987, Nuc. Acids Res. 15:3131-3139. Briefly, tetraethylene glycol was converted to the mono-phthalimido derivative by reaction with phthalimide in the presence of triphenylphosphine and diisopropyl azodicarboxylate (see Mitsunobu, 1981, Synthesis, pp. 1-28). The mono-phthalimide was converted to the corresponding beta-cyanoethyl diisopropylamino phosphoramidite as described in Sinha *et al.*, 1984, Nuc. Acids Res. 12:4539. The resulting phthalimido amidite was added to the 5' ends of the oligonucleotides during the final cycle of automated DNA synthesis using standard coupling conditions. During normal deprotection of the DNA (concentrated aqueous ammonia for five hours at 55 degrees C), the phthalimido group was converted to a primary amine which was subsequently acylated with an appropriate biotin active ester. LC-NHS-biotin (Pierce) was selected for its water solubility and lack of steric hindrance. The biotinylation was performed on crude, deprotected oligonucleotide and the mixture purified by a combination of gel filtration and reversed-phase HPLC (see Levenson *et al.*, 1989, in PCR Protocols and Applications - A Laboratory Manual, eds. Innis *et al.*, Academic Press, NY).

25

After hybridization of the amplified DNA to the membranes and color development, the DQalpha genotypes of these samples is readily apparent, as is shown in Figure 5. In Figure 5, the specificity of each immobilized probe is noted at the top of the filters and the DQA genotype of each sample is noted at the right of the corresponding filter.

30

The immobilized probes of the invention have so facilitated the method of DNA typing at the HLA DQalpha locus that kits for typing will be important commercially. These kits can come in a variety of forms, but a preferred embodiment of the kit is

described in detail, below. This description is followed by a description of simplified typing protocols for use with the kit.

A preferred kit will contain one or more vials of pre-aliquoted, "sterilized" (see below) DQalpha PCR amplification mixes, typically in concentrated (2X is preferred) form and pre-aliquoted in 50 μ l aliquots. Each 50 μ l aliquot will contain: 5 μ mol KCl, 1 μ mol Tris-HCl (pH = 8.3), 250 nmol $MgCl_2$, 15 pmol of biotinylated RS134, 15 pmol of biotinylated RS134, 18.75 nmol each of dGTP, dATP, dTTP, dCTP, and from 2.5 units up to 50 units of recombinant Taq polymerase (PECI). The dNTPs will be prepared from stock solutions at pH = 7. The sterilization protocol also introduces very low levels of inactivated DNase and NaCl, as noted below.

The "sterilized" reagents referred to above relate to the need to avoid contamination of reagents with non-sample-derived nucleic acid sequences. Because PCR is such a powerful amplification method, contaminating molecules can lead to error. To avoid this contamination problem, the present invention provides a novel sterilization procedure. This procedure employs a DNase, preferably bovine pancreas DNase, to remove low levels of DNA contamination from batches of PCR reaction mix. Because DNA primers are sensitive to this enzyme, the primers are omitted from the batch until the DNase has been inactivated by thermal denaturation. However, if RNA primers are to be employed in the PCR mixture, the primers can be present during sterilization. In addition, derivatized nucleotides can be used to make an oligonucleotide resistant to DNase; for instance, thio-substituted nucleotides, such as phosphorothioates can be used to prepare oligonucleotides resistant to DNase (see Sitzler and Eckstein, 1988, *Nuc. Acids Res.* 16:11,691). Those of skill in the art recognize that an equivalent sterilization procedure utilizes a restriction enzyme that cleaves a sequence present in the amplification target; if any contaminating target is present, the restriction enzyme will cleave the contaminant, rendering it unavailable for amplification.

In the preferred sterilization procedure, however, 2.5 ml of 10X Taq buffer (100 mM Tris-HCl, pH = 8.3; 500 mM KCl; and 25 mM $MgCl_2$) are autoclaved and added to 0.19 ml of a solution that is 25 mM in each dNTP, 0.13 ml of Taq DNA polymerase at a concentration of 5 U/ μ l, and 8.75 ml of glass distilled water. The mixing of these reagents can be conveniently carried out in a 50 ml polypropylene tube. Once the mixture is prepared, 650 U of DNase I (Cooper Biomedicals; 2500 U/ml in 150 mM NaCl, stored frozen) are added and the resulting solution incubated at 37 degrees C for 15 minutes. The

DNAse is inactivated by incubating the mixture at 93 degrees C for 10 minutes. Then, 0.38 ml of each primer at 10 μ M is added to the sterilized reagent, which is then aliquoted, preferably with a "dedicated" pipettor and in the protected confines of a laminar flow hood.

The kit can optionally contain the PCR reagents above, but must contain the immobilized probes of the invention, which can be prepared as described above with a blotting and automated pipetting device. The solid support can be conveniently marked by silk-screening. The kit can also contain SA-HRP at a concentration of 20 μ g/ml HRP, which correlates to 250 nmol/ml SA. The SA-HRP is supplied in a buffer composed of 10 mM ACES, 2 M NaCl, at a pH = 6.5. The kit can also contain a concentrated (5X to 20X) solution of chromogen, such as leuco dye (as is marketed by Kodak in the Sure-CellTM diagnostic kits) or TMB.

The kit will also be more successful if simple, easy-to-follow instructions are included. Typical instructions for a preferred embodiment of the present detection method are as follows. About 2 (if hybridization is carried out in a sealed bag) to 3 (if hybridization is carried out in a trough) ml of hybridization solution (5X SSPE, 0.5% SDS, and, in some instances, 1% dextran-sulfate (M.W. 500,000, although other M.W. forms would work) aids in color retention) are pre-warmed to 55 degrees C prior to use. The sample DNA is amplified by PCR using biotinylated primers, and the biotinylated product is heated to 95 degrees for 3 to 5 minutes to denature the DNA. Denaturation can also be accomplished by adding 5 μ l of 5 M NaOH to 100 μ l of PCR product (final NaOH concentration is 250 mM). About 15 μ l of SA-HRP stock (20 μ g/ml, stored at 4 degrees C and never frozen) are then added to the 2 to 3 ml of hybridization solution, and then, 20 μ l of the still hot, denatured PCR product are added to the mixture. If alkali denaturation is used, then one needs to use more PCR product to maintain the same level of sensitivity attained with heat denaturation. Typically 25 to 50 μ l of PCR product are used with 20 to 40 μ l of the SA-HRP stock solution. Best results are obtained when the strepavidin and the biotin are in approximate molar equivalency, i.e., about 300 ng of SA-HRP (measured in HRP) are used for every 6 pmol of biotinylated PCR product used for hybridization. The PCR product should always be added last and immediately after denaturation.

If the hybridization is carried out in a sealed bag, all air bubbles should be removed prior to sealing the bag. If the hybridization is carried out in a trough, the entire trough should be firmly covered with a glass plate. Hybridization is carried out for 20 minutes at 55 degrees C in a shaking water bath set at a moderate to high shaking speed, i.e., 50 to

200 rpm. The wash solution (2X SSPE, 0.1% SDS) is pre-warmed to 55 degrees during the hybridization step. After hybridization, all filters are placed in a bowl containing 200 to 300 ml of pre-warmed wash solution and washed for 8 to 10 minutes in a shaking water bath at 55 degrees C.

Color development is accomplished at room temperature and usually in a shaking water bath as follows if the chromogen is TMB. The filters are rinsed in 200 to 300 ml of room temperature wash solution for 5 minutes, then transferred to 200 to 300 ml of Buffer C (100 mM NaCitrate, pH = 5.0) and rinsed for 5 minutes, then incubated for 5 minutes in 40 ml of Buffer C containing 2 ml of TMB (2 mg/ml in 100% ethanol and stored at 4 degrees C), then transferred to a fresh dye solution (composed of 40 ml of Buffer C and 2 ml of TMB) containing 4 μ l of 30% hydrogen peroxide, and the color is allowed to develop for 5 to 15 minutes. The color development is stopped by rinsing the filters twice with water; the filters can be dried and stored if protected from light. If the typing is weak (faint dots), the procedure is repeated using 50 μ l of the PCR product and 40 μ l of the SA-HRP during the hybridization step. If leuco dye is used in place of TMB, then one replaces the Buffer C rinse with a rinse in 200 to 300 ml of 1X PBS, after which the filters are placed in 25 ml of a mixture of the dye and hydrogen peroxide (the same formulation as in Kodak Sure-CellTM kits). The development time is 5 to 10 minutes; color development is stopped by washing the filters twice in PBS.

Example 5

Detection of Beta-thalassemia Mutations

Although there are over 54 characterized mutations of the beta-globin gene that can give rise to beta-thalassemia, each ethnic group in which this disease is prevalent has a limited number of common mutations (see Kazazian *et al.*, 1984, *Nature* 310:152-154; Kazazian *et al.*, 1984, *EMBO J.* 3:593-596; and Zhang *et al.*, 1988, *Hum. Genet.* 78:37-40). In Mediterranean populations, eight mutations are responsible for over 90% of the beta-thalassemia alleles.

Probes were synthesized that are specific for each of these eight mutations as well as their corresponding normal sequences. The probes were given 400 nt poly-dT tails with terminal transferase and applied to membranes. Various amounts of each probe were applied to twelve duplicate nylon filters, irradiated at 40 mJ/cm², hybridized with amplified beta-globin sequences in genomic DNA samples, and color developed. The result is

shown in Figure 6. In the figure, the beta-thalassemia locus that is detected by each immobilized probe pair is written at the top of the filters. For each filter, the upper row contains the probes that are specific for the normal sequence, and the lower row contains the probes specific for the mutant sequences. The beta-globin genotype of each sample is noted at the right of the corresponding filter. The name, amount applied to the membrane (in pmols and noted parenthetically), specificity, and sequence of each probe is shown below.

| | <u>Probe</u> | <u>Allele Specificity</u> | <u>Sequence</u> |
|----|--------------|------------------------------|------------------------|
| | RS187 (8) | Normal Beta ¹⁻¹¹⁰ | 5'-TAGACCAATAGGCAGAGAG |
| | RS188 (8) | Mutant Beta ¹⁻¹¹⁰ | 5'-CTCTCTGCCTATTAGTCTA |
| 10 | RS87 (4) | Normal Beta ³⁹ | 5'-CCTTGGACCCAGAGGTTCT |
| | RS89 (4) | Mutant Beta ³⁹ | 5'-AGAACCTCTAGGTCCAAGG |
| | RS189 (0.33) | Normal Beta ¹⁻⁶ | 5'-CTTGATACCAACCTGCCA |
| | RS190 (0.33) | Mutant Beta ¹⁻⁶ | 5'-TGGGCAGGTTGGCATCAAG |
| | RS191 (1) | Mutant Beta ¹⁻¹ | 5'-TGGGCAGATTGGTATCAAG |
| 15 | RS192 (4) | Normal Beta ²⁻¹ | 5'-CCATAGACTCACCTGAAG |
| | RS193 (4) | Mutant Beta ²⁻¹ | 5'-CTTCAGGATGAGTCTATGG |
| | RS201 (2) | Normal Beta ²⁻⁷⁴⁵ | 5'-GCAGAATGGTAGCTGGATT |
| | RS202 (2) | Mutant Beta ²⁻⁷⁴⁵ | 5'-GCAGAATGGTACCTGGATT |
| | RS196 (4) | Normal Beta ^{6,8} | 5'-ACTCCTGAGGAGAAGTCTG |
| 20 | RS197 (4) | Mutant Beta ⁶ | 5'-GACTCCTGGGAGAAGTCTG |
| | RS198 (4) | Mutant Beta ⁸ | 5'-TGACTCCTGAGGAGGTCTG |

Because the beta-thalassemia mutations are distributed throughout the beta-globin gene, biotinylated PCR primers that amplify the entire gene in a single 1780 bp amplified product were used. The primers used for the amplification are shown below.

RS151 is 5'-ATCACTTAGACCTCACCTG

RS152 is 5'-GACCTCCCACATTCCCTTTT

This amplification product encompasses all known beta-thalassemia mutations. Following hybridization and color development, the beta-globin genotypes could be determined by noting the pattern of hybridization, as shown in Figure 6.

Unlike the DQalpha typing system, two probes are needed to analyze each mutation – one specific for the normal sequence and one specific for the mutant sequence – to differentiate normal/mutant heterozygous carriers from mutant/mutant homozygotes. A

complicating factor in this analysis is caused by apparent secondary structure in various portions of the relatively long beta-globin amplification product that interferes with probe hybridization. The relatively high stringency needed to minimize this secondary structure requires the use of longer (19 nt hybridizing regions) probes to capture the amplified beta-globin fragment. Because this constraint would not permit varying the length of the probes to compensate for different hybridization efficiencies, the balancing of signal intensities was accomplished by adjusting the amount of each oligonucleotide applied to the membrane.

We Claim:

1. As assay reagent comprising an oligonucleotide probe immobilized on a solid support, said probe comprising a hybridizing region that is a nucleotide sequence complementary to a specific nucleotide sequence to be detected covalently attached to a spacer arm which is longer than said hybridizing region, wherein said probe is immobilized on said solid support by a covalent bond between said solid support and said spacer arm, such that the hybridizing region of said probe can hybridize to said nucleotide sequence to be detected under hybridizing conditions.
2. The assay reagent of Claim 1, wherein said spacer arm is a polynucleotide tail, said solid support contains primary or secondary amines prior to attachment of said probe to said support, and said attachment is formed by ultraviolet light irradiation of said probe on said support.
3. The assay reagent of Claim 2, wherein said probe is an oligodeoxyribonucleotide.
4. The assay reagent of Claim 3, wherein said tail contains from 200 to 800 nucleotides.
5. The assay reagent of Claim 4, wherein said support comprises nylon.
6. The assay reagent of Claim 4, wherein said tail comprises at least 150 pyrimidine nucleotides.
7. The assay reagent of Claim 6, wherein said pyrimidine nucleotides are thymidine nucleotides.
8. The assay reagent of Claim 6, wherein said hybridizing region is a sequence of nucleotides from 17 to 23 nucleotides in length.

- 5 9. The assay reagent of Claim 1 that comprises a set of probes immobilized on a solid support, wherein said set of probes comprises two or more members, each member of said set having a hybridization region different from every other member of said set, wherein each member is immobilized on said solid support at a discrete location separate from every other probe of said set.
10. The assay reagent of Claim 9, wherein each probe of said set of probes is an oligodeoxyribonucleotide.
11. The assay reagent of Claim 9, wherein one member probe of said set serves as a positive control.
- 10 12. The assay reagent of Claim 9, wherein said member probes of said set are complementary to nucleic acid sequences of microorganisms.
13. The assay reagent of Claim 9, wherein said member probes of said set are complementary to variant alleles of a genetic locus.
14. The assay reagent of Claim 13, wherein said genetic locus is an HLA locus.
- 15 15. The assay reagent of Claim 14, wherein said HLA locus is DQalpha.
16. The assay reagent of Claim 9, wherein said spacer arm is a polynucleotide tail, and said solid support contains primary or secondary amines prior to attachment of said probe to said support, and said attachment is formed by ultraviolet light irradiation of said probe on said support.
- 20 17. A method for preparing the assay reagent of Claim 2, which method comprises: (a) contacting said probe with a solid support comprising amine groups; and (b) irradiating the support prepared in step (a) with ultraviolet light.

18. A method for detecting the presence of a nucleic acid sequence in a sample, which method comprises: (a) contacting said sample with the assay reagent of Claim 1 under conditions that allow for hybridization of complementary nucleic acid sequences; and (b) determining if hybridization has occurred.

5 19. A kit comprising the assay reagent of Claim 1 and instructions for detecting specific nucleic acids with said reagent.

20. An assay reagent comprising a set of oligonucleotide probes covalently attached to a solid support, wherein said set of probes comprises two or more members, each member of said set having a hybridization region different from every other member
10 of said set, wherein each member is immobilized on said support at a discrete location separate from every other probe of said set.

21. The reagent of Claim 20, also comprising a labeled polynucleotide hybridized to one of said probes, wherein said polynucleotide comprises at least 50 nucleotides.

15 22. The reagent of Claim 20, also comprising a target sequence from a sample hybridized to an immobilized probe of said set and a colored or fluorescent compound immobilized on said support at the location of said hybridized probe.

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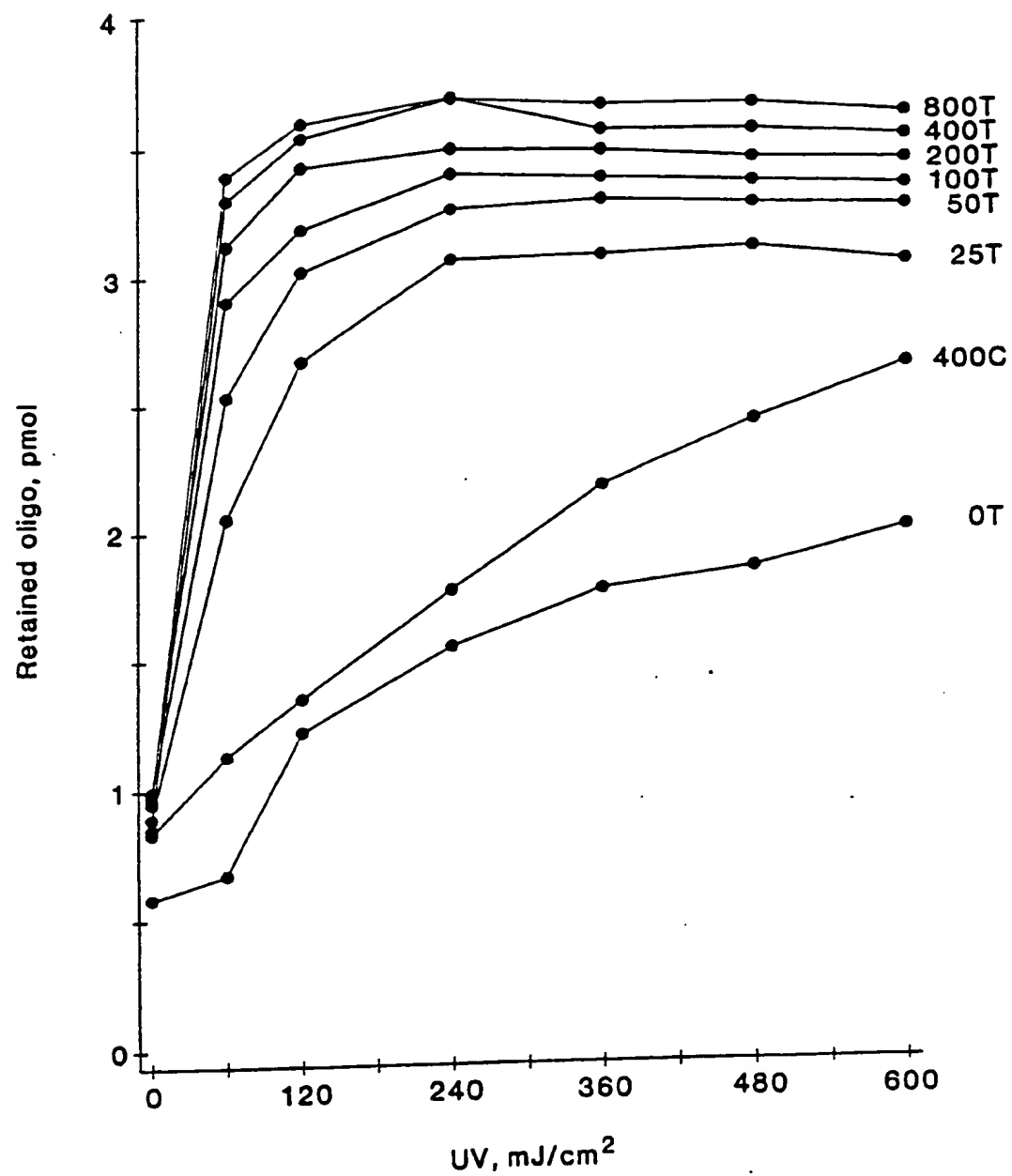


FIG. 1

SUBSTITUTE SHEET

2 / 6

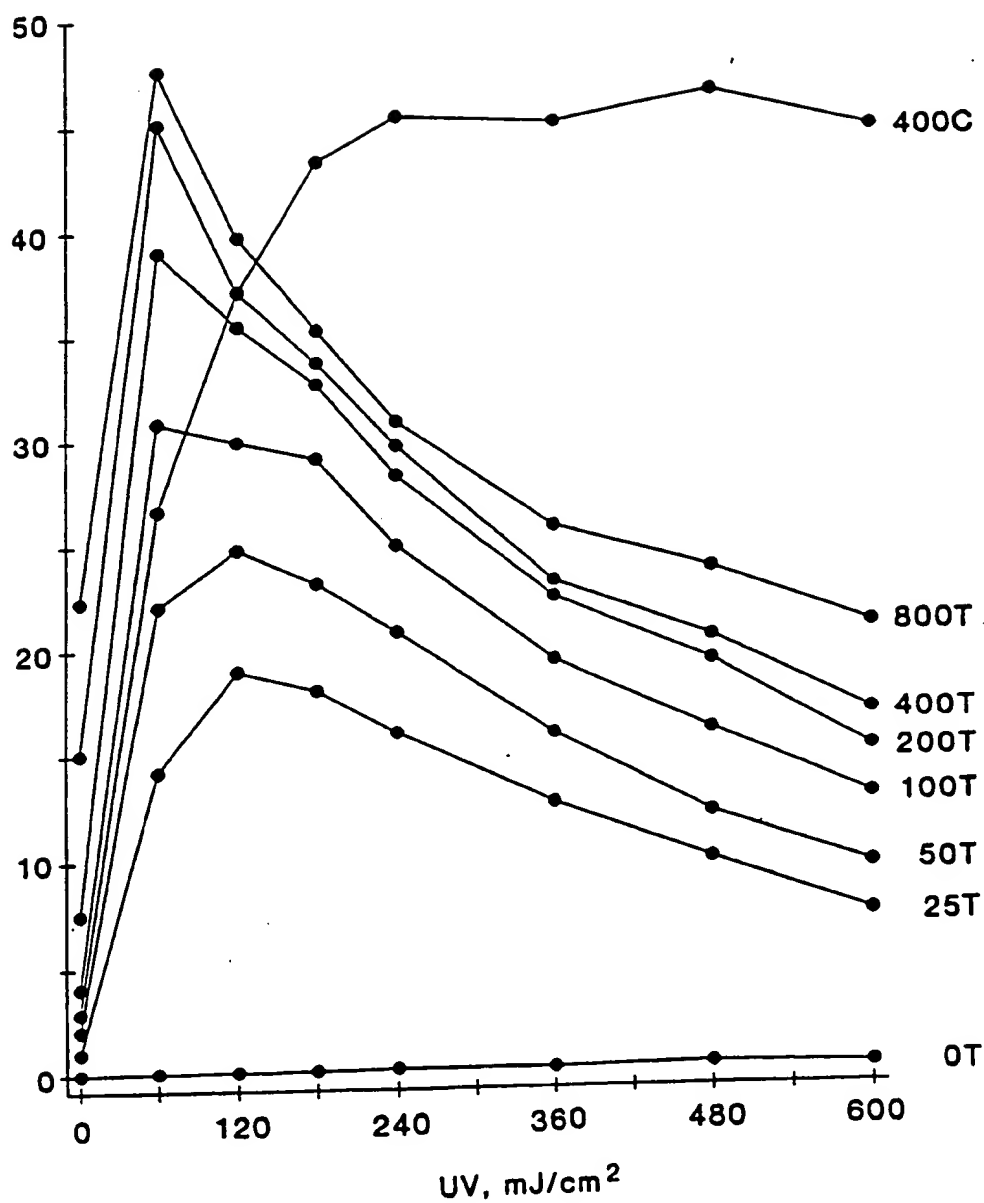


FIG. 2

SUBSTITUTE SHEET

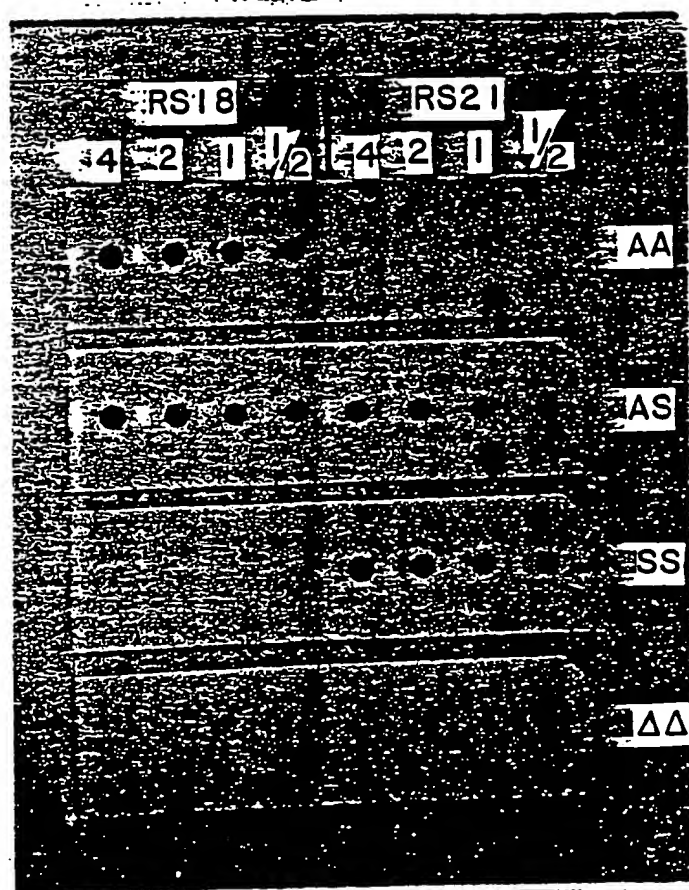


FIG. 3

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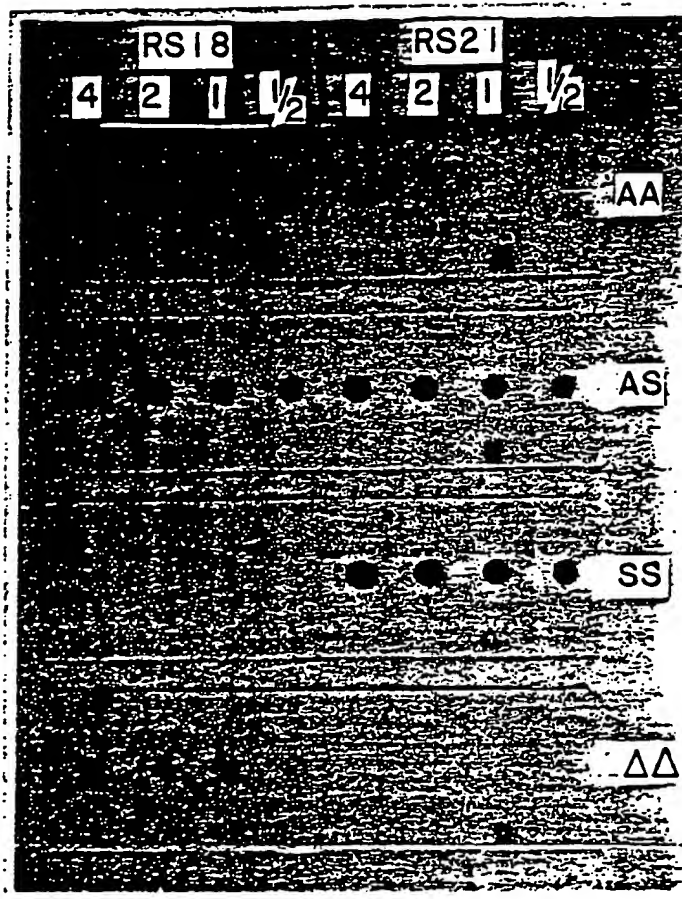


FIG. 4

| All | DQA 1 | DQA 2 | DQA 3 | DQA 4 | DQA 1.1 | DQA 1.2, 1.3, 4 | DQA 1.3 | not DQA 1.3 | |
|-----|-------|-------|-------|-------|---------|-----------------|---------|-------------|-------------|
| ● | ● | | | | ● | | | ● | DQA 1.1/1.1 |
| ● | ● | | | | | ● | | ● | DQA 1.2/1.2 |
| ● | ● | | | | | ● | ● | | DQA 1.3/1.3 |
| ● | | ● | | | | | | ● | DQA 2/2 |
| ● | | | ● | | | | | ● | DQA 3/3 |
| ● | | | | ● | | ● | | | DQA 4/4 |
| | | | | | | | | | |
| ● | | | | | | | | | DQA 2/4 |
| ● | | | | | | ● | | | DQA 1.2/4 |
| ● | | | | | | | | ● | DQA 1.2/3 |
| ● | | ● | | ● | | ● | | ● | DQA 2/4 |
| ● | ● | | | | ● | | | ● | DQA 1.1/2 |
| ● | | | ● | ● | | ● | | ● | DQA 3/4 |

FIG. 5

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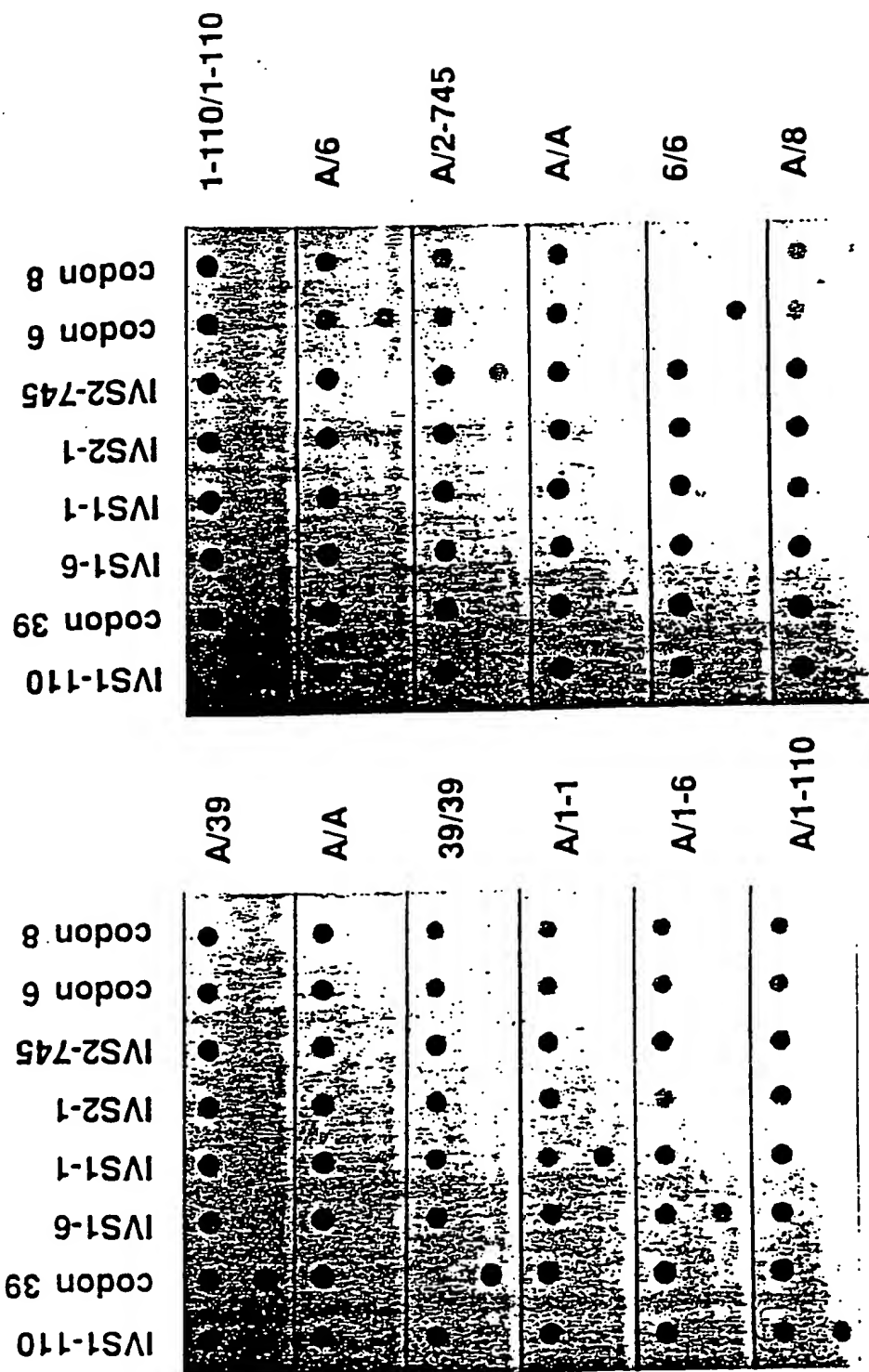


FIG. 6

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/02170

| I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) * According to International Patent Classification (IPC) or to both National Classification and IPC IPC ⁴ : C 12 Q 1/68 | | | | | | | | | | | | | | | | | | | | |
|---|--|-------------------------------------|---|---|--|--|--|----------------------------------|---|----|------|---|--|-----------|---|--------------------------------|------|---|--|------|
| II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Minimum Documentation Searched ⁷</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 30%; border-bottom: 1px solid black; padding: 2px 5px;">Classification System</td> <td style="border-bottom: 1px solid black; padding: 2px 5px;">Classification Symbols</td> </tr> <tr> <td style="padding: 5px 5px 0 5px;">IPC⁴</td> <td style="padding: 5px 5px 0 5px;">C 12 Q 1</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸</div> | | | Classification System | Classification Symbols | IPC ⁴ | C 12 Q 1 | | | | | | | | | | | | | | |
| Classification System | Classification Symbols | | | | | | | | | | | | | | | | | | | |
| IPC ⁴ | C 12 Q 1 | | | | | | | | | | | | | | | | | | | |
| III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹ <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%; padding: 2px;">Category *</th> <th style="width: 60%; padding: 2px;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 30%; padding: 2px;">Relevant to Claim No. ¹³</th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="padding: 5px;">EP, A, 0130523 (MOLECULAR DIAGNOSTICS INC.) 9 January 1985, see page 2, lines 2-33; page 3, lines 17-31; page 13, lines 32-34; page 14, lines 29-30; page 16, line 27 - page 17, line 2</td> <td style="vertical-align: top; padding: 5px;">1-4, 6, 8, 10, 13, 14, 16-19, 22</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="text-align: center; vertical-align: top; padding: 5px;">--</td> <td style="vertical-align: top; padding: 5px;">1-22</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="padding: 5px;">Proceedings of the National Academy of Sciences USA, vol. 81, April 1984, (Washington, D.C., US), G.M. Church et al.: "Genomic sequencing", pages 1991-1995, see the whole article cited in the application</td> <td style="vertical-align: top; padding: 5px;">5, 13, 14</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;">cited in the application --</td> <td style="vertical-align: top; padding: 5px;">1-22</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="padding: 5px;">Tetrahedron Letters, vol. 22, no. 34, 1981, Pergamon Press Ltd (GB), I. Saito et al.: "Photochemical ring opening of thymidine and thymine in ./. .</td> <td style="vertical-align: top; padding: 5px;">6, 7</td> </tr> </tbody> </table> | | | Category * | Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹² | Relevant to Claim No. ¹³ | X | EP, A, 0130523 (MOLECULAR DIAGNOSTICS INC.) 9 January 1985, see page 2, lines 2-33; page 3, lines 17-31; page 13, lines 32-34; page 14, lines 29-30; page 16, line 27 - page 17, line 2 | 1-4, 6, 8, 10, 13, 14, 16-19, 22 | Y | -- | 1-22 | X | Proceedings of the National Academy of Sciences USA, vol. 81, April 1984, (Washington, D.C., US), G.M. Church et al.: "Genomic sequencing", pages 1991-1995, see the whole article cited in the application | 5, 13, 14 | Y | cited in the application -- | 1-22 | X | Tetrahedron Letters, vol. 22, no. 34, 1981, Pergamon Press Ltd (GB), I. Saito et al.: "Photochemical ring opening of thymidine and thymine in ./. . | 6, 7 |
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| Y | -- | 1-22 | | | | | | | | | | | | | | | | | | |
| X | Proceedings of the National Academy of Sciences USA, vol. 81, April 1984, (Washington, D.C., US), G.M. Church et al.: "Genomic sequencing", pages 1991-1995, see the whole article cited in the application | 5, 13, 14 | | | | | | | | | | | | | | | | | | |
| Y | cited in the application -- | 1-22 | | | | | | | | | | | | | | | | | | |
| X | Tetrahedron Letters, vol. 22, no. 34, 1981, Pergamon Press Ltd (GB), I. Saito et al.: "Photochemical ring opening of thymidine and thymine in ./. . | 6, 7 | | | | | | | | | | | | | | | | | | |
| <div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>[*] Special categories of cited documents: ¹⁰</p> <p>^{"A"} document defining the general state of the art which is not considered to be of particular relevance</p> <p>^{"E"} earlier document but published on or after the international filing date</p> <p>^{"L"} document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>^{"O"} document referring to an oral disclosure, use, exhibition or other means</p> <p>^{"P"} document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>^{"T"} later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>^{"X"} document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>^{"Y"} document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>^{"&"} document member of the same patent family</p> </div> </div> | | | | | | | | | | | | | | | | | | | | |
| IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;"> Date of the Actual Completion of the International Search <div style="border: 1px solid black; padding: 2px; display: inline-block;">27th September 1989</div> </td> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;"> Date of Mailing of this International Search Report <div style="border: 1px solid black; padding: 2px; display: inline-block;">L</div> </td> </tr> <tr> <td style="border-bottom: 1px solid black; padding: 5px;"> International Searching Authority <div style="border: 1px solid black; padding: 2px; display: inline-block;">EUROPEAN PATENT OFFICE</div> </td> <td style="border-bottom: 1px solid black; padding: 5px;"> Signature of Authorized Officer <div style="text-align: right; padding-top: 10px;"> T.K. WILLIS </div> </td> </tr> </table> | | | Date of the Actual Completion of the International Search <div style="border: 1px solid black; padding: 2px; display: inline-block;">27th September 1989</div> | Date of Mailing of this International Search Report <div style="border: 1px solid black; padding: 2px; display: inline-block;">L</div> | International Searching Authority <div style="border: 1px solid black; padding: 2px; display: inline-block;">EUROPEAN PATENT OFFICE</div> | Signature of Authorized Officer <div style="text-align: right; padding-top: 10px;"> T.K. WILLIS </div> | | | | | | | | | | | | | | |
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| III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET) | | |
|--|---|----------------------|
| Category * | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No |
| | the presence of primary amines", pages 3265-3268, see the whole article cited in the application -- | |
| Y | EP, A, 0142299 (FUJIREBIO INC.) 22 May 1985, see page 3, lines 24-28; page 2, lines 22-37 -- | 1-21 |
| Y | Nucleic Acids Research, vol. 14, no. 24, 1986, IRL Press Ltd (Oxford, GB), H.B. Gamper et al.: "Reverse southern hybridization", pages 9943-9954, see the whole article ---- | 1-21 |

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.

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SA 29159

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| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---------------------|----------------------------|---------------------|
| EP-A- 0130523 | 09-01-85 | US-A- 4542102 | 17-09-85 |
| | | US-A- 4713326 | 15-12-87 |
| | | AU-B- 563558 | 16-07-87 |
| | | AU-A- 3025684 | 10-01-85 |
| | | CA-A- 1215703 | 23-12-86 |
| | | DE-A- 3471689 | 07-07-88 |
| | | JP-A- 60036496 | 25-02-85 |
| EP-A- 0142299 | 22-05-85 | JP-A- 60091999 | 23-05-85 |

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